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Pathogenesis of the *Helicobacter* Induced Mucosal Disease: A Dissertation

Calin Stoicov
University of Massachusetts Medical School

PATHOGENESIS OF THE *HELICOBACTER* INDUCED MUCOSAL DISEASE

A Dissertation Presented

By

CALIN STOICOV, MD

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CALIN STOICOV, MD

Approved as to style and content of the Dissertation by:

_____
JeanMarie Houghton MD/PhD, Thesis Advisor

_____
Evelyn Kurt-Jones PhD, Member of Committee

_____
Andrew Leiter MD/PhD, Member of Committee

_____
Steven Moss MD, Member of Committee

_____
Andre van Wijnen PhD, Member of Committee

_____
Stephen Lyle, MD/PhD, Chair of Committee

_____
Anthony Carruthers Ph.D, Dean of the
Graduate School of Biomedical Sciences

Millennium MD/PhD Program
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DEDICATION

THIS THESIS IS DEDICATED TO MY DEAR WIFE DIANA, MY LOVELY CHILDREN DAN AND MADALINA; TO MY PARENTS MARIA AND MIHAI STOICOV.

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ABSTRACT

Helicobacter pylori causes chronic gastritis, peptic ulceration and gastric cancer. This bacterium is one of the most prevalent in the world, but affects mostly the populations with a lower socioeconomical status. While it causes gastric and duodenal ulcers in only 20% of infected patients, less then 1% will develop gastric adenocarcinoma. In fact, *H. pylori* is the most important risk factor in developing gastric cancer. Epidemiological studies have shown that 80% of gastric cancer patients are *H. pylori* positive. The outcome of the infection with this bacterium depends on bacterial factors, diet, genetic background of the host, and coinfection with other microorganisms. The most important cofactor in *H. pylori* induced disease is the host immune response, even though the exact mechanism of how the bacterium is causing disease is unknown.

The structural complexity of *Helicobacter* bacteria makes us believe that different bacterial factors interact with different components of the innate immunity. However, as a whole bacterium it may need mainly the TLR2 receptor to trigger an immune response. The type of adaptive immunity developed in response to *Helicobacter* is crucial in determining the consequences of infection. It is now known for decades that a susceptible host will follow the infection with a strong Th1 immune response. IFNγ, IL-12, IL-1β and TNF-α are the key components of a strong adaptive Th1 response. This is further supported by our work, where deficient T-bet (a master regulator for Th1 response) mice were protected against gastric cancer, despite maintaining an infection at similar levels to wild type mice. On the other hand, a host that is resistant to *Helicobacter* develops an infection that is followed by a Th2 response sparing the mucosa from severe

inflammation. Human studies looking at single nucleotide polymorphism of cytokines, like IL-1β, IL-10 and TNF-α have clearly demonstrated how genotypes that result in high levels of IL-1β and TNF-α, but low IL-10 expression may confer a 50-fold higher risk in developing gastric cancer.

The outcome of *Helicobacter* infection clearly relies on the immune response and genetic background, however the coinfection of the host with other pathogens should not be ignored as this may result in modulation of the adaptive immunity. In studying this, we took advantage of the Balb/C mice that are known to be protected against *Helicobacter* induced inflammation by mounting a strong Th2 polarization. We were able to switch their adaptive immunity to Th1 by coinfected them with a *T. gondii* infection (a well known Th1 infection in mice). The dual infected mice developed severe gastritis, parietal cell loss and metaplastic changes. These experiments have clearly shown how unrelated pathogens may interact and result in different clinical outcomes of the infected host.

A strong immune response that results in severe inflammation will also cause a cascade of apoptotic changes in the mucosa. A strict balance between proliferation and apoptosis is needed, as its disruption may result in uncontrolled proliferation, transformation and metaplasia. The Fas Ag pathway is the leading cause of apoptosis in the *Helicobacter*-induced inflammation. One mechanism for escaping Fas mediating apoptosis is upregulation of MHCII receptor. Fas Ag and MHCII receptor interaction inhibits Fas mediated apoptosis by an impairment of the Fas Ag receptor aggregation when stimulated by Fas ligand. Because *H. pylori* infection is associated with an upregulation of the MHCII levels on gastric epithelial cells, this indeed may be one mechanism by which cells escape apoptosis.

The link between chronic inflammation and cancer is well known since the past century. *Helicobacter* infection is a prime example how a chronic inflammatory state is causing uncontrolled cell proliferation that results in cancer. The cell biology of “cancer” is regarded not as an accumulation of cells that divide without any control, but rather as an organ formed of cancer stem cells, tumor stromal support cells, myofibroblasts and endothelial cells, which function as a group. The properties of the cancer stem cells are to self-renew and differentiate into tumor cells thus maintaining the tumor grow, emphasizing that a striking similarity exists between cancer stem cells and tissue stem cells.

We looked into what role would BMDCs play in chronic inflammation that causes cancer. Using the mouse model of *Helicobacter* induced adenocarcinoma we discovered that gastric cancer originates from a mesenchymal stem cell coming from bone marrow. We believe that chronic inflammation, in our case of the stomach, sets up the perfect stage for bone marrow stem cells to migrate to the stomach where they are exposed to inflammatory stimuli and transform into cancer stem cells. One of the mechanism by which the MSC migrate to the inflammation site is the CXCR4/SDF-1 axis.

Our work sheds new light on *Helicobacter* induced gastric cancer pathogenesis. I hope that our findings will promote the development of new therapies in the fight against this deadly disease.

ABREVIATIONS

AIB-1 - amplified in breast cancer 1

APC - adenomatous polyposis coli gene

APC - antigen presenting cell

BMDC - bone marrow–derived cells

BrdU - bromodeoxyuridine

C-MET - (MNNG (N-Methyl-N'-nitro-N-nitroso-guanidine) HOS transforming gene

Cag - cytotoxin-associated gene

cAMP - cyclic adenosine monophosphate

CDX2 - caudal-related homeobox 2

CFU - colony forming units

CIITA – major histocompatibility class II transactivator

COX2 - cyclooxygenase-2

CpG - cytosine-phosphate-guanosine

CYP - cytochrome P450

CytoD - cytochalasin D

DAPI - 4',6-diamidino-2-phenylindole

DC - dendritic cell

DISC - death-inducing signal complex

ELISA - enzyme-linked immunosorbent assay

ERK - extracellular signal regulate kinases

FACS - fluorescence-activated cell sorter

FADD - Fas-Associated protein with Death Domain

FAP-1 - Fas associated phosphatase -1

FCS - fetal calf serum

FHIT - fragile histidine triad gene

FISH - fluorescent in situ hybridization

FITC - fluorescein isothiocyanate

Fla - flagellin

FLIP - FLICE inhibitory protein

GalR2 - galanin receptor-2

GAPDH - glyceraldehyde-3-phosphate dehydrogenase

GFP - green fluorescent protein

GI - gastrointestinal

GIF - gastric intrinsic factor

GIN - gastrointestinal intraepithelial neoplasia

Git1 - G-protein-coupled receptor kinase interactor 1

GST - glutatione S-transferases

H. felis - Heliocobacter felis

H. pylori - Helicobacter pylori

H&E - hematoxyline&eosin

HGF/SF - hepatocyte growth factor/scatter factor

HK ATPase - hydrogen potassium ATPase

HP-NAP - neutrophil-activating protein

HSC - hematopoietic stem cells

IFNγ - interferon gamma

IHC - immunohistochemistry

Ii – invariant chain

IL - interleukin

IM - intestinal metaplasia

INS-GAS - insulin-gastrin transgenic mouse

KO - knockout

KRT1-19 - keratine 1-19

LPS - lipopolysaccharide

MALT - Mucosa-Associated Lymphoid Tissue

MCP1 - monocyte chemotactic protein-1

MHCII - major histocompatibility class II

MIP1α - Macrophage Inflammatory Protein 1 alpha

MKK - MAP (Mitogen-Activated Protein) Kinase Kinase

MMP1 - matrix metalloproteinase one

MSC - mesenchymal stem cells

MUC2 - mucin 2

MyD88 - myeloid differentiation 88

NFAT - nuclear factor of activated T cells

NOD1 - nucleotide-binding oligomerization domain 1

ODN - oligodinucleotides

PAS - periodic acid-Schiff

PBS - phosphate buffered saline

PBST - phosphate buffered saline Tween

PE - phycoerythrin

PGE - prostaglandin E

PI - propidium iodine

Ptprz - protein tyrosine phosphatase receptor type Z

RAG - recombinase activating gene

RANTES - regulated upon activation, normal T cell expressed and secreted

RFP - red fluorescent protein

RGM-1 - rat gastric mucosal cells

RUNX3 - runt-related transcription factor 3

SCID - severe combined immunodeficiency

SDF-1 – stromal derived factor 1

SMA - smooth muscle actin

SNPs - single nucleotide polymorphisms

SP - spasmolytic polypeptide

SPEM - spasmolytic expressing metaplasia

SRC - sarcoma proto-oncogenic tyrosine kinase

STAT4 - Signal Transducers and Activators of Transcription 4

T-BET - Th1-specific T box transcription factor

TA - transient amplifying

TFF2 - trefoil factor 2

TLR - Toll-like receptor

TNF-α - tumor necrosis factor alpha

VacA - vacuolating cytotoxin A

VEGF - vascular endothelial growth factor

WHO - World Health Organization

WT - wild type

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CHAPTER I: INTRODUCTION TO *HELICOBACTER* INDUCED DISEASES AND PATHOGENESIS

1. Gastric cancer: classification and epidemiology.

Gastric adenocarcinoma is the second most common cause of cancer-related mortality worldwide and the 14th overall cause of death (1). There are widespread geographical differences in gastric cancer rates with the highest rates seen in Japan, China and South America, and much lower rates in Western Europe and the United States (1). The single most common cause of gastric cancer is infection with *Helicobacter pylori*: one of the most common chronic bacterial infections in man (2). This bacterium colonizes over half of the world's population. Infection is usually acquired in childhood and in the absence of antibiotic therapy persists for the life of the host (3).

H. pylori is a member of a large family of related bacteria that colonize the mammalian stomach, and is causally linked to chronic gastritis, peptic ulceration, gastric atrophy, gastric adenocarcinoma and rarely non-Hodgkin lymphoma of the stomach (4). In the United States, the rate of *H. pylori* acquisition is less than 1% per year, with 10–15% of children less than 12 years of age and 50–60% of people greater than 60 years of age infected (5).

The gastric environment is relatively inhospitable to bacteria and other pathogens. As a result of harsh pH conditions, the presence of digestive enzymes and constant contractions with sweeping motility designed to clear the gastric contents at regular intervals, the stomach is a relatively sterile organ. Indeed, *H. pylori* is the only known bacteria that can persistently colonize the normal stomach, and essentially sets up a monoculture within the gastric mucosa.

Although most infected patients will develop a chronic active gastritis, the

majorities are asymptomatic. Though the risk varies with age, geographical location and ethnicity, overall 15–20% of infected patients will develop gastric or duodenal ulcer disease and less than 1% will develop gastric adenocarcinoma (4, 6). Unfortunately, symptoms and disease are poorly correlated, and many cases of gastric cancer are detected late in disease progression, when the disease is incurable.

The pattern of gastritis has been shown to correlate strongly with the risk of gastric adenocarcinoma. Patients who develop antral-predominant gastritis (the most common manifestation) are at a higher risk of developing duodenal ulcers, while those that develop corpus-predominant gastritis and multifocal atrophic gastritis are at a higher risk of developing gastric ulcers, and of progressing to intestinal metaplasia, dysplasia and adenocarcinoma (7). Noncardia gastric cancers (Lauren classification) are divided into two histological distinct variants termed intestinal-type adenocarcinoma and diffuse-type adenocarcinoma (8). Intestinal-type gastric adenocarcinoma forms gland-like structures and primarily involves the distal stomach (9). The development of the intestinal type of gastric carcinoma appears to progress through a stepwise transition from normal mucosa through atrophic gastritis, atrophy, and intestinal metaplasia. It is in the setting of atrophy and metaplasia that dysplasia arises, which may progress to adenocarcinoma (10), though the point of transition from a “premalignant” to “malignant” lesion has not been identified. Intestinal-type adenocarcinoma has a predilection for the elderly of lower socioeconomic status and has a male-to-female ratio of 2:1.

In contrast, the histology of diffuse type of gastric cancer is one of individual neoplastic cells infiltrating the gastric mucosa, without forming glandular structures. There is no association with atrophy or intestinal metaplasia, and there is no stepwise

progression as seen in intestinal-type cancer (11). The diffuse type of gastric cancer presents at a younger age, affects men and women equally and there is a stronger genetic component.

2. *Helicobacter* infection in the initiation and promotion of gastric cancer.

H. pylori has been classified by the World Health Organization (WHO) as a class 1 carcinogen, although the precise mechanism by which this bacterium causes gastric cancer is not clear (12). The evidence supporting a role for *H. pylori* causing gastric cancer is very strong. Epidemiological studies link *Helicobacter* colonization with noncardia gastric cancer (13, 14), with the decline in *H. pylori* infection in industrialized counties in the last century correlating closely with decreased morbidity and mortality from gastric adenocarcinoma (15, 16). Case controlled studies have established a positive link between seropositivity and gastric adenocarcinoma; with a 2.1- to 16.7- fold increase risk when compared to seronegative patients (14, 17).

More recently, the presence of past or present *Helicobacter* infection has been confirmed in the majority (up to 80%) of gastric cancer patients, demonstrating conclusively that *Helicobacter* infection is associated with the majority of gastric cancer cases (18, 19). The precise manner in which infection causes cancer is not known; however, the present thinking is that cancer arises through the combination of infection with a virulent organism, a permissive environment and a genetically susceptible host. Candidates for cancer induction include direct *H. pylori* bacterial factors, components of the host immune response, dietary cofactors including high salt and decreased ascorbate,

gastrin hormonal levels, and decreased acid secretion with bacterial overgrowth. Indeed, all these factors may interact to alter host cell signaling, derange apoptotic and proliferative signaling, and promote the acquisition and maintenance of genetic mutations leading to neoplasia.

2.1. H. pylori bacterial factors.

H. pylori is well adapted to the gastric environment, withstanding low pH to gain entry to its preferred niche, the mucus layer of the stomach. Once there, the bacterium evades local and systemic immune responses that are ineffective at eliminating the organism. In the absence of antibiotic therapy, colonization persists for the life of the host. The *H. pylori* genome has 1.65 million base pairs and codes for approximately 1500 genes (20). Of these, only 2/3 of the genes have been assigned biological roles. The function of the remaining 1/3 of the genome remains obscure. A modified signature tagged mutagenesis approach, used to detect the genes essential for colonization and survival of *H. pylori* identified 47 genes, including several previously known factors such as urease and motilin, thereby validating this method (2). Bacterial factors, which contribute to carcinogenesis, include those that enable the bacteria to effectively colonize the gastric mucosa, incite a more aggressive host immune response and direct virulence factors of the organism.

2.2. Motility.

Motility toward epithelial cells of the stomach is a vital feature of *H. pylori*

survival tactics. This function is assured by several factors including spiraling movement designed to navigate the thick gastric mucus and through efficient modifications of the extracellular matrix and mucus layer thus decreasing viscosity and allowing bacterial penetration. The complex flagella filament is composed of two flagellin subspecies, the more abundant *flaA* protein, which comprises the majority of the filament, and a second slightly larger protein, *flaB*, which appears to be exclusively located proximal to the hook within the assembled filament (21) (Fig. 1.1A). Both are essential for flagella integrity and function to quickly propel the bacterium from the hostile gastric lumen, to the protected niche of mucus overlying the gastric mucosal cells. Once safely out of the lumen, the bacterium modifies the local environment to allow unhampered movement over the surface of epithelial cells. A gene coding for a putative *collagenase (HP0169)* enzyme has been described (2). This enzyme is actively transported to the bacterial cell surface where it may remain, or is secreted into the extracellular space and functions to digest collagen, allowing freer bacterial movement. Collagen types III and I are important components of the extracellular matrix of the gastric epithelium. Secretion by gastric cells is induced in areas of ulceration and gastritis where it functions to promote healing. Through digestion of collagen, collagenase secreted by *H. pylori* may be responsible for delay of ulcer healing and chronic ulcers. Additionally, components of the mucosal immune response, such as IgA antibodies, may be degraded by bacterial collagenase further contributing to disease by *Helicobacter* or other transient organisms within the GI (gastrointestinal) tract (2) (Fig. 1.1A).

FIGURE 1.1 *H. PYLORI* BACTERIAL VIRULENCE FACTORS.

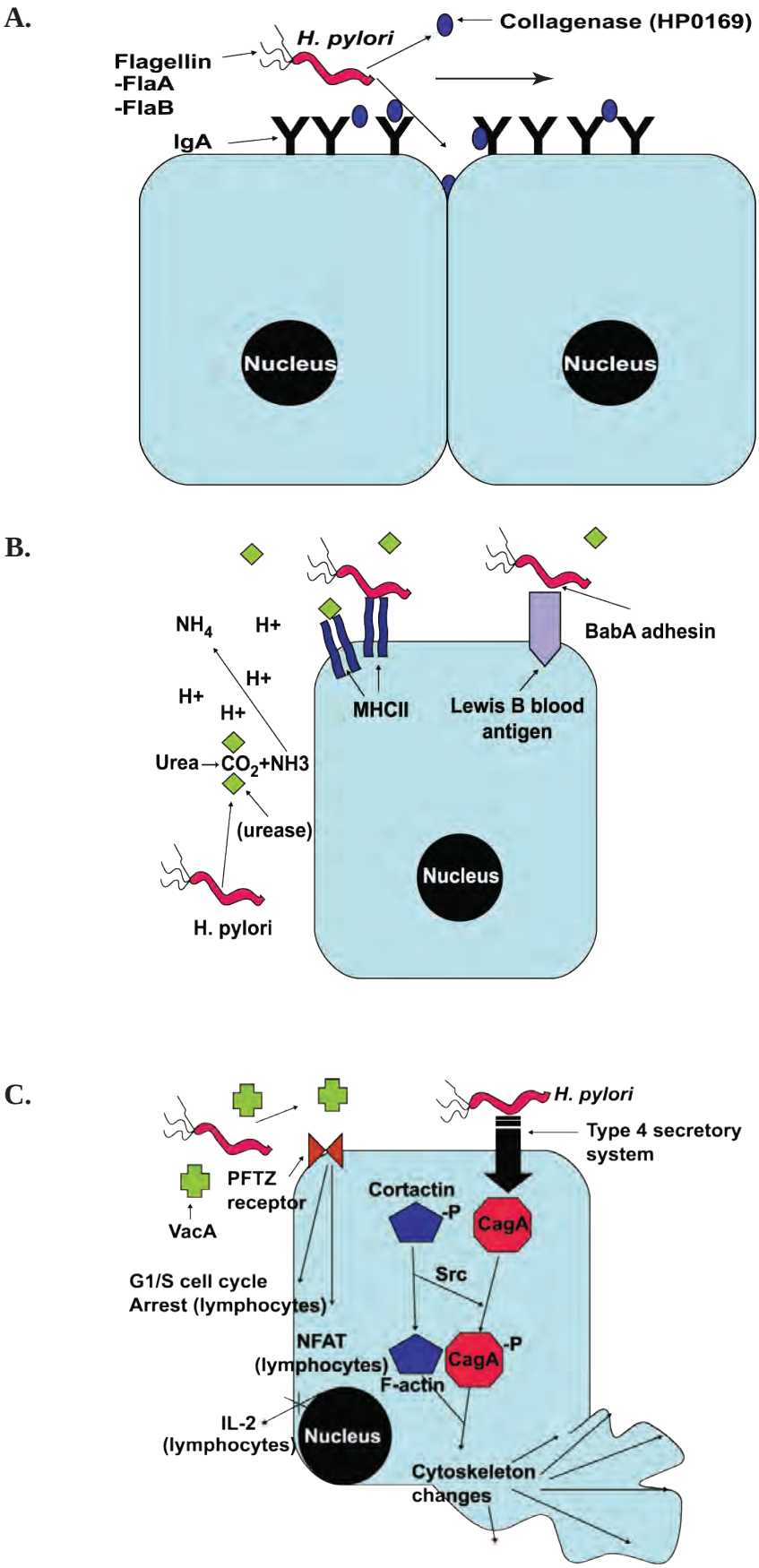


FIGURE 1.1 *H. PYLORI* BACTERIAL VIRULENCE FACTORS.

(A) Helicobacter gains access to the epithelial cell surface through a combination of spiral motility provided by powerful flagella composed of subunits flagellin A and B (FlaA and FlaB), and secretion of a collagenase (Hp 0169) which acts to decrease viscosity and allow bacterial movement over the mucosal cell surface. Collagenases degrade other host molecules such as immunoglobulin A (IgA) favoring bacterial – host cell attachment. **(B)** Once in contact with the mucosal surface, bacteria can adhere to the epithelial cells via an adhesin (BabA) - Lewis B blood group antigen. Urease, produced by the bacterium, converts urea to CO2 and NH3, buffering the mucus niche creating a neutral pH habitat directly over the cell surface. **(C)** Using a type IV secretory system, *Helicobacter* proteins and DNA are introduced into host cells. Once inside, CagA (cytotoxin-associated gene A) can be phosphorylated by host proteins. Phosphorylated CagA, in turn, dephosphorylates host proteins, altering cytoskeletal structure and interfering with cell signaling. VacA (vacuolating cytotoxin A) within the cell leads to T-lymphocyte inactivation through G1/S cell cycle arrest and targets the nuclear factor of activated T-cells (NFAT) signaling pathway to hinder cytokine signaling.

2.3. Adhesion factors.

Adhesins are a large family of 32 related outer membrane proteins (Hop proteins) that assures adhesion of the bacteria to the epithelial layer. One of the best-characterized adhesin is *babA*, encoded by the strain specific gene *babA2*, a member of a highly conserved family of outer membrane proteins. *babA* binds to the fucosylated Lewis B blood group antigen on gastric epithelial cells and forms a scaffold apparatus that allows bacterial proteins to enter host epithelial cells. Bacterial strains which posses the *babA2* gene adhere more tightly to epithelial cells and promote a more aggressive phenotype, and are associated with a higher incidence of gastric adenocarcinoma. Transgenic mice carrying the Lewis B blood group antigen challenged with *babA+ H. pylori* strains are more likely to develop severe gastritis, atrophy and antiparietal cell antibodies than the same mice challenged with wild-type strains of bacteria (22), via increased bacterial adherence to mucosal cells. On the other hand, there are some *H. pylori* strains that produce low levels of *babA* and have a low binding activity to the gastric mucosa; however these strains some times may have a higher likelihood to cause gastric inflammation and atrophy. The underlying reason is unclear and needs further investigations (23) (Fig. 1.1B).

2.4. Urease.

Urease is produced by all strains of *H. pylori* and functions to hydrolyze urea into CO2 and NH3. NH3 effectively buffers the acid environment immediately surrounding the bacterium permitting survival in the acid environment of the stomach. Urease enzyme

activity is tightly controlled by a pH-gated urea channel (*ureI*), which is open at low pH and closed at neutral pH conditions (24), allowing the bacterium a precise level of control over its pH environment (Fig. 1.1B).

2.5. The cag (cytotoxin-associated gene) pathogenicity island.

The most important feature, which distinguishes strains of *H. pylori* is the presence or absence of the *cag* island. The Cag pathogenicity island is approximately 40 kb, and contains 31 genes. The terminal gene of this island, *cagA*, is often times used as a marker for the entire *cag* locus. Several genes within the island have homology to genes that encode a type IV secretory system. Once the bacteria adhere to the gastric epithelial cell, the type IV secretion system functions to export bacterial DNA and/or proteins into the host cell cytoplasm. In vitro experiments suggest that once in the cytoplasm, *cagA* undergoes kinase-mediated phosphorylation. Phosphorylated *cagA* in turn dephosphorylates host cell proteins, activating intracellular signaling pathways. Alterations in host cell proteins lead to actin-cytoskeleton reorganization and morphological changes described in cultured cells as the "hummingbird phenotype".

To further study this phenomenon in vivo, Ohnishi et al. generated a wild type *cagA* and a phosphorylation resistant *cagA* transgenic mouse. The transgenic mouse with wild type *cagA* developed impressive gastric and small intestine adenocarcinoma. However, the phosphorylation resistant *cagA* transgenic mice did not develop these pathological findings. This is one of the first evidences of *cagA* being a bacterial oncoprotein acting in mammals (25). Compared to *cagA*- strains, *cagA*+ strains are

associated with more severe inflammation, higher degrees of atrophic changes and a greater chance of progressing to gastric adenocarcinoma (13, 26-31). Other genes within the pathogenicity island are felt to be important for disease (*cagE* or *picB*, *cagG*, *cagH*, *cagI*, *cagL*, *cagM*), as they appear to be required for in vitro epithelial cell cytokine release (32-34), though there does not appear to be as great an effect on immune cell cytokine activation. These findings may explain the attenuated inflammatory response and lower cancer risk with *cagA*- strains in vivo (28, 35-37) (Fig. 1.1C)**.**

All strains of *H. pylori* carry the *vacA* (vacuolating cytotoxin A) gene, which codes for a vacuolating toxin, with expression varying depending on allelic variation. Approximately 50% of *H. pylori* strains express the vacuolating cytotoxin A (*vacA* protein). Although *vacA* and *cagA* map to different loci within the *H. pylori* genome, they commonly are found together. The vacuolating toxin, composed of identical 87 kDa monomers assembled into a flower-shaped oligomer, alters intracellular vesicular trafficking in eukaryotic cells, leading to formation of large vacuoles (38). When administered orally to mice, *vacA* alone is able to induce gastric epithelial erosions, resembling erosions found in *Helicobacter*-infected humans (39).

It is proposed that VacA binds protein tyrosine phosphatase receptor type Z (*Ptprz*), increases the tyrosine phosphorylation of the G-protein-coupled receptor kinase interactor 1 (*git1*, a Ptprz substrate), inducing mucosal damage. In support of this, the endogenous ligand of Ptprz, pleiotropin, induces gastritis in wild-type mice. Ptprz-/- mice do not develop *vacA*-induced mucosal damage, even though *vacA* successfully incorporates into gastric epithelial cells (40). The challenge of Ptprz-/- mice with “full” *H. pylori* was lacking in this study, which would have confirmed the physiological

significance of these findings. In addition to effects on gastric epithelial cells, *vacA* cytotoxin acts to inhibit T lymphocyte activation through induction of a G1/S cell cycle arrest, impedes transcription of IL-2 and downregulates IL-2R-alpha through targeting the nuclear factor of activated T cells (NFAT) signaling pathway (41) at the level of calcineurin (42). Additionally, *vacA* has been shown to activate MKK3/6 (MAP (Mitogen-Activated Protein) Kinase Kinase), p38, and a *rac*-specific nucleotide exchange factor (*vav*) by presumed interaction with an as of yet unknown receptor (40). This aberrant activation of *rac* stimulates actin polymerization, altering cellular architecture and interfering with the formation of the immunological synapse between antigen-presenting cells (APCs) and T cells (43) (Fig. 1.1C). These effects impair T lymphocyte activation and perpetuate an ineffective immune response, permitting chronic colonization of the stomach.

Other bacterial virulence factors, such as *cagE*, may play a role in the modulation of apoptosis and the host inflammatory response thereby contributing to disease manifestations. Indeed, “virulent strains” (*cagA*+, *cagE*+ and *vacA*+) appear to be more potent inducers of pro-inflammatory mediators than “nonvirulent strains” (*cagA*-, *cagE*- and *vacA*-) possibly explaining the higher association of *cag*+ strains with gastric cancer (4).

H. pylori neutrophil-activating protein (HP-NAP) is another major virulence factor, which was initially described as promoter of neutrophil adhesion to the endothelial cells and it is known to stimulate the production of oxygen radicals by neutrophils (44). Amedei et al. have found HP-NAP to be a TLR2 (Toll-like receptor) agonist, which by stimulating the neutrophils increase their expression of IL-12. Furthermore, when HP-

NAP stimulates the monocytes it will induce their production of IL-23, and differentiate them towards a mature dendritic cell. A T–cell that is undergoing a specific antigen challenge has the propensity to polarize towards a Th-1 phenotype, when stimulated with HP-NAP in vitro and in vivo (45).

3. Host immune response - an overview.

Evidence to date clearly shows the most important cofactor in the induction of *Helicobacter*-related disease is the host immune response. Indeed, chronic inflammation has been linked to a number of gastrointestinal cancers including chronic viral hepatitis and hepatocellular carcinoma; Barrett's adenocarcinoma of the esophagus arising in the setting of chronic reflux disease; and colonic adenocarcinoma arising in the setting of chronic inflammatory bowel disease, thus setting the stage for the investigation of chronic inflammation in the pathogenesis of gastric adenocarcinoma. How the bacterium initiates an immune response is not precisely known and likely involves multiple factors. Despite the unclear initiating events, it is certain that chronic inflammation is necessary for the progression through atrophy to gastric cancer. I will begin by defining what we know about the initial events in immune recognition of the bacterium, and outline how the polarity and strength of the adaptive response impacts disease outcomes.

3.1 The innate immune response to H. pylori in initial bacterial recognition.

Initiation of the innate immune response to *H. pylori* is just beginning to be

unraveled. Recent work supports a role for pattern recognition receptors (Toll-like receptors or TLRs) in the initial response to *Helicobacter* colonization, though there is not yet a consensus as to which receptor within this group is most important, or which cell type expressing the receptor plays the central role in antigen recognition (Fig. 1.2)

Helicobacter produces a very weak LPS (lipopolysaccharide): up to 10,000- fold less active than LPS of other gram-negative bacteria, such as *Echerichia coli* (46, 47). We know that LPS from gram-negative bacteria, such as *E. coli*, predominantly stimulate innate immunity through TLR4 (48). Data regarding *H. pylori* LPS are contradictory, with reports of activation of both TLR4 (49) and TLR2 (50) in gastric epithelial cells. These contradictions may be explained by differences in bacterial strain, different bacterial concentrations used or trace contaminants in the LPS preparations such as peptidoglycan or lipopeptides, both which signal via TLR2 receptor. While the cell type responsible for initiation of the immune response has not been identified, it is possible that the gastric immune cells themselves act as the first line of recognition. Gastric epithelial cells reportedly express TLR4, 5 and 9 on their surface (49). In addition to LPS acting locally, it may gain access to the blood stream to be detected by peripheral blood monocytes (51).

Whole *Helicobacter* bacteria (*H. pylori*, *H. hepaticus* and *H. felis*) activate immune response by TLR2, not TLR4 (52), suggesting various components of the bacterium (i.e. LPS, proteins, flagella) may activate various members of the TLR family. Indeed, expression of human TLR2 has been shown to be sufficient to confer responsiveness to intact *Helicobacter* bacteria, whereas TLR4 was not. Macrophages from both wild-type and TLR4-deficient mice produce a robust cytokine secretion

FIGURE 1.2 INNATE IMMUNITY IN *HELICOBACTER* INFECTION.

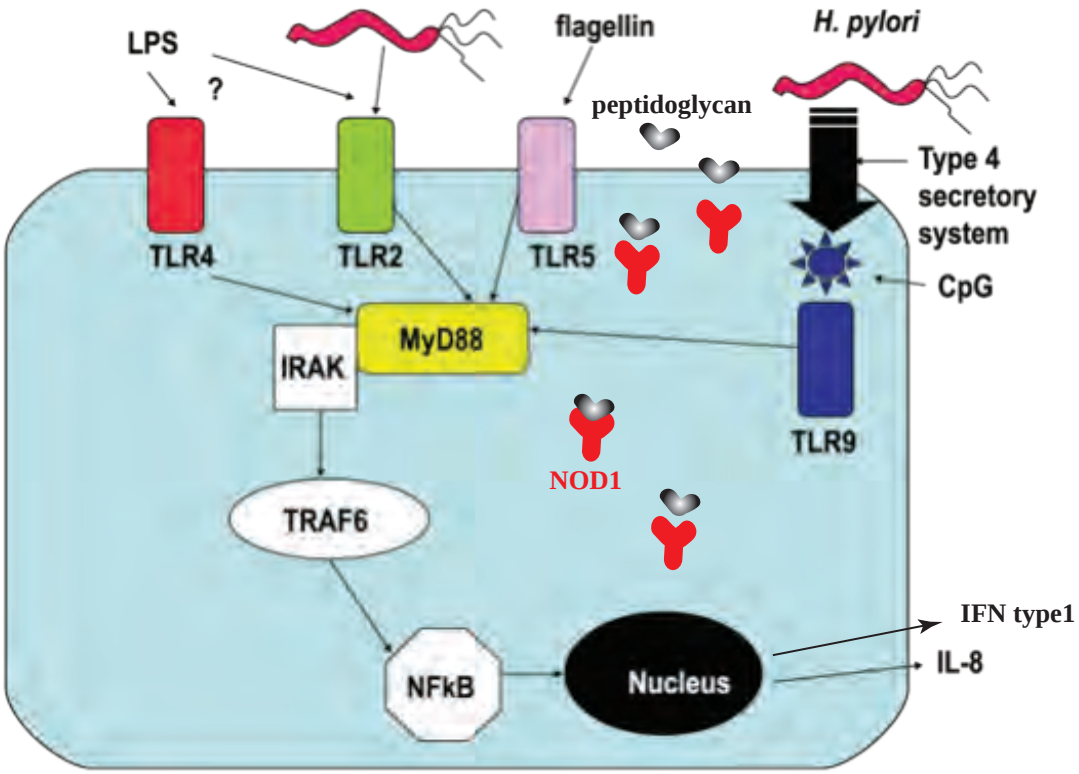


FIGURE 1.2 INNATE IMMUNITY IN *HELICOBACTER* INFECTION.

Multiple members of the family of Toll-like receptors (TLR) have been implicated in innate recognition of *Helicobacter* species. LPS (lipopolysaccharide) may activate the immune response through TLR2 and/or TLR4. Whole bacteria appear to be recognized by TLR2 exclusively. TLR5 may recognize bacterial flagella. Bacterial DNA containing CpG islands (high frequency CpG sites in DNA) may be recognized by intracellular TLR9. *Helicobacter* peptidoglycans may bind the intracytoplasmic NOD1 (nucleotide-binding oligomerization domain) and activate interferon type 1 pathway.

response (IL-6 and MCP1 (Monocyte chemotactic protein-1)) when stimulated with intact *Helicobacter* bacteria, while macrophages from TLR2-deficient mice were profoundly unresponsive to intact bacteria, failing to secretion cytokines even at high (100:1) bacteria-to-macrophage ratios. Because the quantity and strength of bacterial LPS is so weak, the involvement of TLR4 in clinical disease is unclear; in vitro and in vivo studies suggest that TLR2 may be the dominant innate immune receptor for recognition of gastrointestinal *Helicobacter* species (52). TLR5 recognizes flagellin proteins; however, a role for TLR5 in *Helicobacter* recognition is not clear. Gastric mucosal cells in culture express low but detectable amounts of TLR5 (53). TLR9 recognizes CpG (cytosine-phosphate-guanosine) molecules and distinguishes bacterial DNA from vertebrate DNA (54), inducing a very potent systemic Th1 response as a result (Fig. 1.2). CpG oligodinucleotides (ODNs) may be phagocytosed by antigen-presenting cells (APCs) or injected directly into the cytoplasm using the bacterial type IV secretory system. Interaction between TLR9 and CpG molecules intracellular recruits the MyD88 (myeloid differentiation primary response gene 88) adapter protein and triggers a strong Th1 immune response (55).

TLR signaling is required only for Th1 type of adaptive immunity, not for the development of Th2 type lymphocyte subsets. Th2 cells are most likely activated by another distinctive (but presently unknown) pathway. In the absence of induced IL-12 production by dendritic cells, a Th2 response appears to the “default” pathway (56).

H. pylori peptidoglycan also activates the intracellular cytoplasmic nucleotide-binding oligomerization domain (NOD) 1 in gastric epithelial cells promoting a Th1 response (57). NOD domains are linked to a leucine-rich repeat domain at the C-terminus

and to a caspase-recruitment domain (CARD) at the N-terminus. NOD1 and its family member, NOD2 recognize peptidoglycans of the bacterial cell wall. NOD1 is expressed in APCs and most of the gastrointestinal epithelial cell proving their role as a defense mechanism in the gastrointestinal tract (Watanabe T. 2010 in press). It is known that NOD1 activation induces a potent IL-8 secretion in the epithelial cells of the stomach (58).

Recently, Watanabe et al. have demonstrated clearly that NOD1 activation by *Helicobacter* is using its type IV secretion system. This stimulates IP-10 secretion, which attracts Th1 cells to the infected mucosa, resulting in epithelial cells and incoming Th1 cells to produce IFN-γ in large amounts that will enhance the magnitude of the inflammatory response (Watanabe T. 2010 in press) (Fig. 1.2).

3.2 Adaptive immunity to Helicobacter infection.

Genetic manipulation of immune cell constituents has been used to evaluate the role of the inflammatory response in the pathogenesis of gastric cancer. For example, infection in recombinase activating gene (RAG)-deficient mice, severe combined immunodeficiency (SCID), and T cell deficient mice fail to produce tissue damage or recreate the metaplasia–dysplasia–carcinoma sequence (59, 60). In contrast, infection in B cell deficient mice (which retain a normal T cell response) is indistinguishable from infection in the wild type mice, stressing a crucial role for CD4 T lymphocytes in orchestrating disease (62). In order to understand what it is about the CD4 T lymphocyte

that is related to disease, Th1/Th2 patterns were evaluated between susceptible and resistant strains. A susceptible strain (such as the C57BL/6 mice) is able to mount a strong immune response (Th1) which results in mucosal disease (61, 62), while a resistant strain (such as the Balb/C mice) develops an infection, but responds with a polarized Th2 response and appear protected from mucosal damage despite maintaining bacterial colonization (61). Interestingly, strains such as the C3H, which have a mixed Th1/Th2 cytokine profile, develop a mild mucosal disease. Although the composite immune picture most likely determines how disease is manifest, there may be a role for individual cytokines in both the predisposition to and protection from disease.

More recently, Shi et al. have described elegantly how *H. pylori* infection promotes the secretion of IL-17, which results in the development of a Th17 immune response. Th17 will modulate the Th1 polarization in response to H. pylori, helping in bacterial colonization and resulting in gastric mucosal inflammation (63).

Several studies have examined the role of individual cytokines in disease progression. For example, the IFN-γ knockout mouse is protected from *Helicobacter*-induced atrophy (61, 62), while IFN-γ infusion into the infected C57BL/6 mouse accelerates disease progression (64). In contrast, the IL-10 knockout develops severe atrophic gastritis (61, 62), possibly due to the inability to modulate the immune response. While these studies provide useful information, one must be cautious in interpreting the data. The effect of over- or underexpressing a single cytokine has widespread impact on other signaling pathways, and effects may be secondary and not due to direct action of the cytokine that has been manipulated. To further address this issue, others and we have

manipulated the immune response within wild-type strains. For example, infection with the intestinal helminth, *Heligmosomoides polygyrus,* skews the immune response toward Th2 polarization and protects the C57BL/6 host from *Helicobacter* induced atrophy and preneoplasia (65). Conversely, our group published how induction of a Th1 response in the Balb/C induces atrophy, metaplasia and dysplasia (66) and converts this formerly resistant strain to a susceptible host **(CHAPTER IV).**

In humans, the designation of an immune response as Th1 or Th2 is somewhat artificial and simplistic as the adaptive response in humans can more accurately be described as falling somewhere on a continuum between these two extremes.

The first cytokine studied in detail in humans was interleukin-1β, a pro-inflammatory cytokine shown to induce gastrin release, inhibit acid secretion, and promote apoptosis. Studies by El-Omar looking at single nucleotide polymorphisms (SNPs) within the IL-1β gene suggested that high-expressing IL-1β genotypes increase the risk of developing both atrophy and gastric cancer secondary to *Helicobacter* infection (67). A combination of IL-1β, TNF-α (tumor necrosis factor alpha) and IL-10 SNPs, which potentially result in a phenotype of elevated IL-1β and TNF-α; and decreased IL-10 has been shown to confer a 50-fold increased risk of gastric cancer (68, 69). More recently, Erzin et al. analyzed a cohort of 93 *H. pylori* positive Turkish patients in tandem with their host genetic factors and established that bacterial factor babA2 is the most important predictor of a malignant outcome of an *H. pylori* infection, but the presence of the IL-1β-31T/T genotype is as a protective factor against it (70). There is also a synergistic effect between IL-10-592A/A and IL-8-251A/A in regards to

developing gastric cancer, after an analysis of a cohort of 1187 Korean patients (71).

Recent evidence describes the role of other cytokines in the *H. pylori* pathogenesis. For example, following an *H. pylori* infection, gastric epithelial cells will produce IL-18, a potent Th-1 cytokine. Patients infected with *Helicobacter pylori* who have a high IL-18 expression genotype (IL-18-607C/C and IL-18-137G/G) will develop a more severe gastritis (72).

4. Inflammation, stem cells and carcinoma development.

Helicobacter pylori has gone from an unknown organism ”contaminating” pathology slides to the celebrity of the past century. Once passed over, it is now recognized as the leading cause of gastric cancer worldwide, largely through its effects on the host immune response. Thus *Helicobacter* has become an important tool for the study of inflammation-induced cancers, and has provided for us a glimpse into the turmoil of chronically infected tissues. Over the last several years there has been a resurgence of interest in the association of inflammation and cancer. While inflammation was initially felt to be a beneficial response, we now recognize that in many situations inflammation may in fact drive malignancy.

The concept of a cancer stem cell, or a cancer-initiating cell has regained attention in recent years. What years ago started as a theory, is now supported by solid experimental data derived from multiple models and multiple cancer types. It is believed that all tumors contain a subset of cells (termed the cancer stem cell or cancer initiating cell) responsible for the growth, differentiation, invasion and metastasis of the tumor.

The exact proportion of these cells within the tumor itself is controversial and likely varies widely. The surface marker profile and gene expression pattern of these cells is beginning to be delineated, and while there is seemingly a different signature for each tumor type evaluated, there are also significant similarities, suggesting a common origin may exist for these cells. Now that we recognize a cancer stem cell as the soul of a tumor- responsible for it's very existence- we must address the next question- from where does this cancer stem cell originate? Throughout the history of cancer research, virtually every cell type has at one time or another been considered as a candidate for the cancer-initiating cell. Our recent thinking however assigns this task to a cell with inherent progenitor or stem cell function. I will discuss the history of the cancer stem cell hypothesis, and present the arguments and data to support a role for a bone marrow derived cell as an additional candidate cell type to the more widely held peripheral stem cell candidate.

The traditional model of cancer development suggests that genetic instability and/or environmental factors affect the differentiated cells of the tissue, inducing mutations that will lead to carcinogenesis. The tumor cell progresses through preneoplastic into a neoplastic stage that may, at later times, metastasize (73, 74). The majority of cancer research in the last century has focused on tumor cell properties, with less attention dedicated to understanding what type of cells are affected by these mutations, leaving the question – what is the source of the cancer stem cell- unanswered.

In order to understand where cancer stem cells come from, we must first understand the potential sources for this cell, and the growth properties inherent of each of these cell types.

Most tissues in the mammalian body have a population of adult stem cells or progenitor stem cells. Tissue specific stem cells have two main properties: the ability of self-renewal and the ability to differentiate into all cell types of the tissue of origin. Adult stem cells are believed to be capable of an unlimited number of cell divisions. During cell division, the stem cell is thought to divide asymmetrically, producing an identical daughter stem cell, which remains relatively quiescent, and a transit-amplifying cell. The transit-amplifying (TA) cell is highly proliferative and gives rise to various differentiated mature cell types specific to the tissue they reside in. These unique biological properties of the adult stem cell allows it to supply the tissues indefinitely with mature differentiated cells, ensuring homeostatic control in the face of continuous turnover, such as occurs in the gut, skin, blood etc., yet delegating the actual work of proliferation to a more "transient" cell (TA) (75). The majority of DNA damage is acquired during cell replication, putting a replicating cell population at risk for malignant transformation. This burst of proliferative activity in a cell with a finite life span may ensure cell proliferation while maintaining the safe guard of TA elimination prior to accumulating genetic damage. In most tissues the true stem cells are rare and relatively quiescent, making them less likely to accumulate genetic damage, but also making them difficult to prospectively identify and study. While the progenitor cell is considered a strong candidate in becoming the first cancer stem cell, it remains unclear if this is still valid when its population is completely exhausted in a severe inflammation case.

The other potential source of stem cells is the bone marrow. Within the bone marrow, there are at least two populations of stem cells, hematopoietic and mesenchymal stem cells (known as bone marrow derived cells, BMDCs). The hematopoietic stem cell is

responsible for producing all the formed elements of the blood. The mesenchymal stem cell was originally recognized as essential for the production of stromal support cells necessary for hematopoiesis, and later recognized as having tri-lineage potential, with the ability to differentiate to bone, cartilage and fat. The mesenchymal stem cell has been shown to possess the plasticity to differentiate down most all cell lineages in the body and participate in tissue restoration and healing as epithelial cells as well as stromal cells. Under normal physiologic conditions, multiple types of epithelial cells are shown to be derived from bone marrow cells, including epithelium of the lung, gastrointestinal tract and skin (76, 77). These data strongly support the existence of a single pluripotent stem cell rather than multiple committed progenitor cells as the cell of origin. Thus, bone marrow is an excellent candidate for a continuous resource of stem cells.

Taking advantage of the chronic infection of C57BL/6 mice with *Helicobacter* as a model of chronic inflammation and carcinogenesis, we will examine if BMDCs have any role in promoting gastric malignancy **(CHAPTER VI)**.

5. Alterations in gastric mucosal signaling: regulation of apoptotic and proliferative pathways.

The host response to infection induces multiple changes within the gastric mucosa leading to the formation of adenocarcinoma. The balance between apoptosis (Fig. 1.3) and proliferation (Fig. 1.4) is severely altered and the cellular composition distorted as parietal and chief cells are depleted and metaplastic lineages emerge. These changes are likely due to effects of Th1 type cytokines, which act at several levels including induction

FIGURE 1.3 APOPTOTIC PATHWAYS IN *HELICOBACTER* INFECTION.

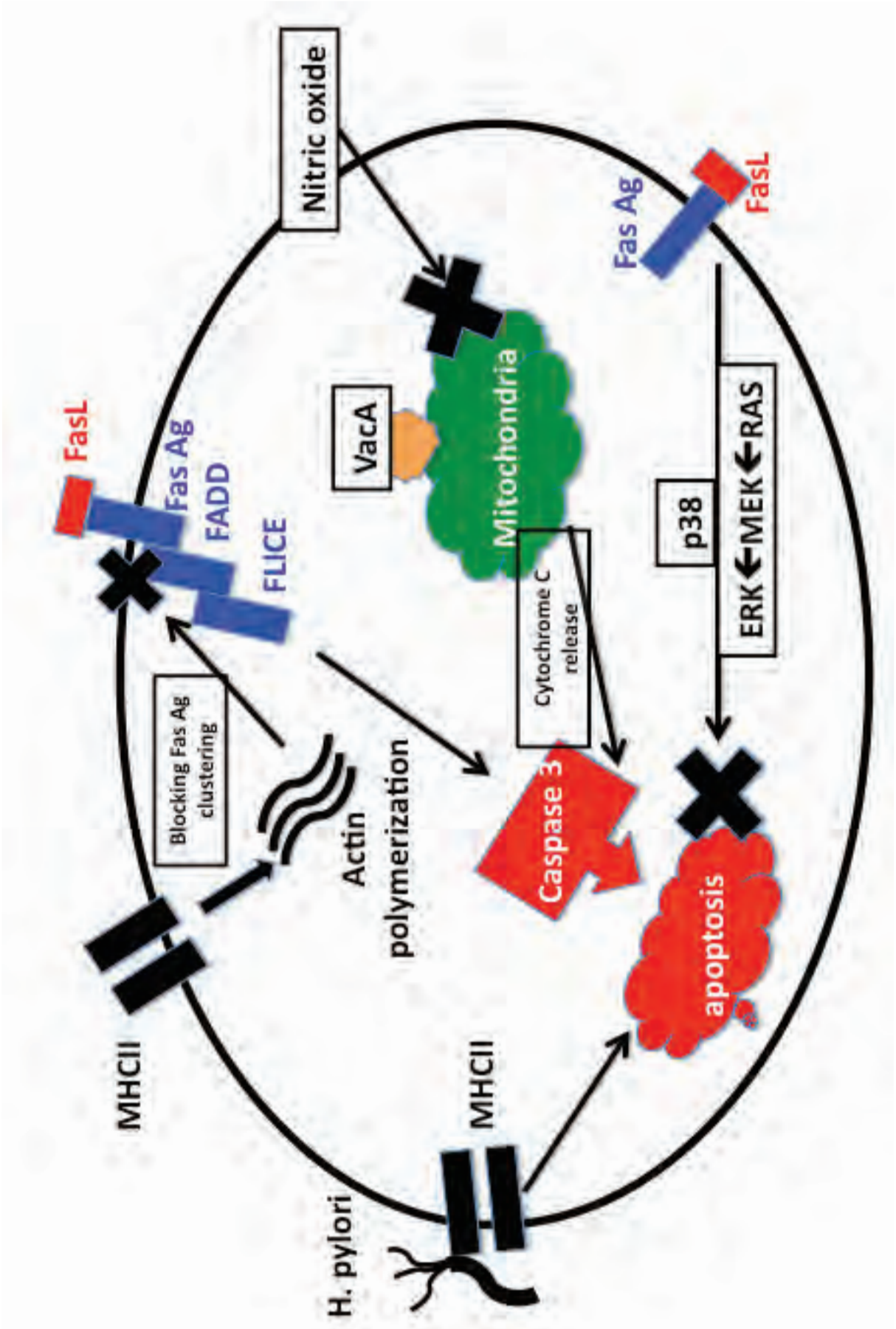


FIGURE 1.3 APOPTOTIC PATHWAYS IN *HELICOBACTER* INFECTION. Several pathways for initiation of apoptosis, and of apoptosis control are active during *Helicobacter* infection. Fas Ag on the mucosal cell surface, and FasL on invading immune cells are upregulated by inflammatory cytokines. The Fas Ag pathway may be inhibited by major histocompatibility class II (MHCII) expression, which impairs Fas Ag receptor aggregation and DISC (death-inducing signaling complex) formation, via nitric oxide (NO) -induced nitrosylation of caspases, and through p38 and kinase Ras/MEK/ERK (mitogen-activated protein kinase /extracellular signal–regulated kinases) inhibition of apoptosis downstream of the Fas receptor. *Helicobacter* may bind to MHCII directly and induce apoptosis. VacA may directly induce cytochrome c release from mitochondria, inducing apoptosis.

FIGURE 1.4. PROLIFERATION PATHWAYS IN *HELICOBACTER* INFECTION.

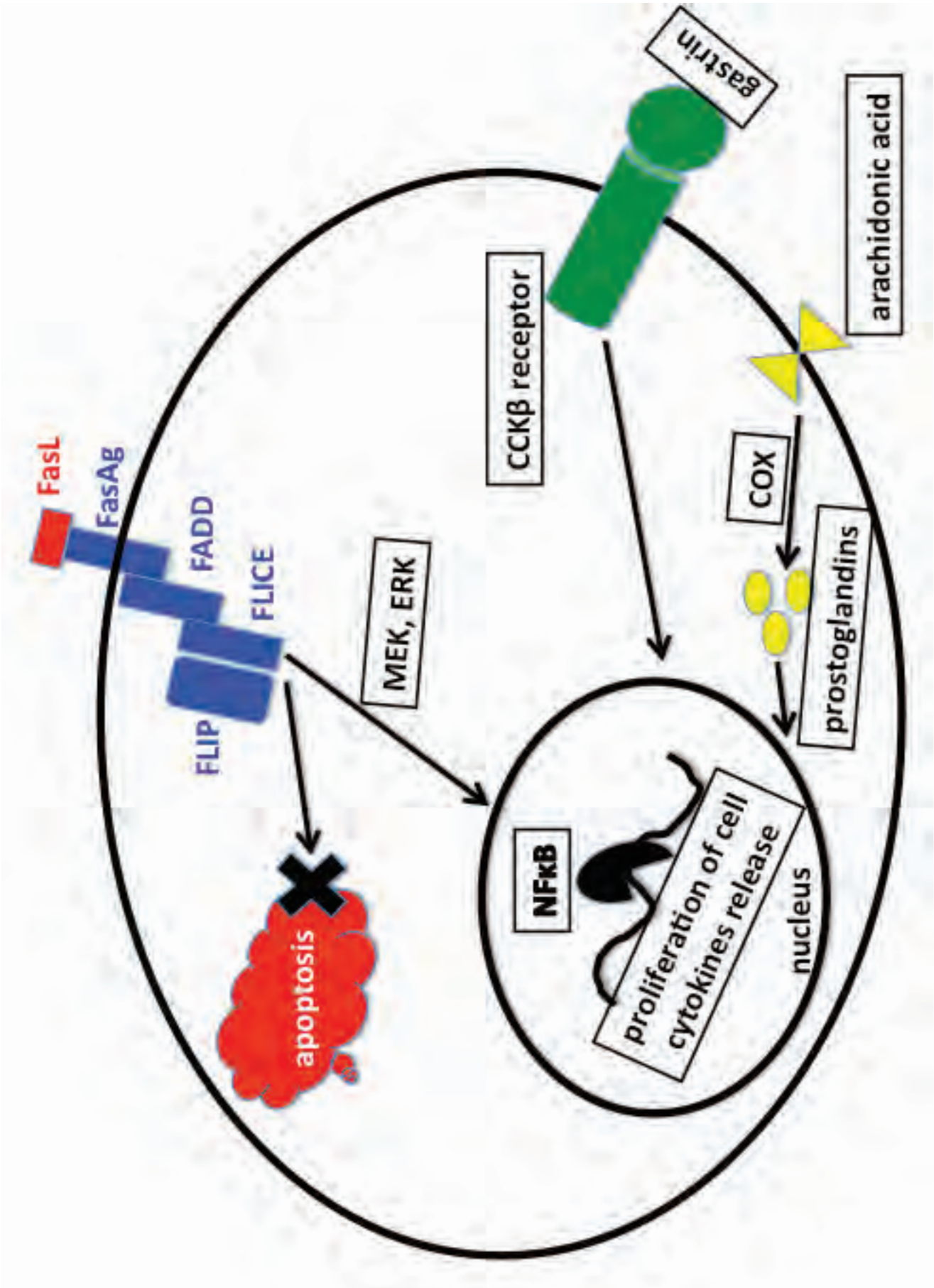


FIGURE 1.4 PROLIFERATION PATHWAYS IN *HELICOBACTER* INFECTION.

Infection with *Helicobacter* organisms can induce host cell proliferation through several pathways, including Fas-mediated proliferation through MEK/ERK (mitogen-activated protein kinase /extracellular signal–regulated kinases) activation of NFκB (nuclear factor κ-light-chain-enhancer of activated B cells); activation of the MAPK/ERK pathway separate from Fas signaling, through the effects of the hormone gastrin, or via the arachidonic acid pathway.

of hypochlorhydria and atrophy (68), direct cytokine signaling, or secondary effects through modulation of other growth promoting signaling cascades. *H. pylori* in humans has been associated with both an increase and a decrease in apoptosis, dependent upon the population studied (78, 79). Increase in proliferation is seen in all cases of mucosal damage due to infection. Apoptosis and proliferation are intimately associated, and regulations of these pathways are more effectively discussed together. Apoptosis is a physiological safeguard against perpetuating acquired DNA damage. While increased apoptosis in the setting of *Helicobacter* infection may contribute to ulcer formation and cell lineage depletion, inhibition of apoptosis may lead to transformation of cells leading to gastric adenocarcinoma. Mouse models of infection combined with human and rat cell culture systems have provided a great deal of insight into the interactions of the bacterium with various host cell proliferative and apoptotic pathways and have allowed detailed study of individual signaling cascades not possible in human systems.

The Fas Ag pathway of apoptosis is recognized as a leading cause of tissue destruction during *Helicobacter* infection. More recently, however, a role for Fas Ag signaling in proliferation has been recognized (80, 81). *H. pylori* can both directly and indirectly (through cytokine production) induce Fas Ag and Fas ligand expression (78, 82-84). In transformed cell lines, *H. pylori* can directly induce and activate the Fas pathway (83). In nontransformed cell cultures, direct bacterial contact does not appear sufficient for induction and activation, but requires components of the immune response. Our laboratory has shown that IL-1β, TNF-α and IFN-γ are produced during infection increase surface Fas Ag expression on gastric mucosal cells resulting in increased

proliferation at low receptor abundance, and increased susceptibility to Fas L-induced apoptosis at high receptor abundance (80, 85).

In the wild-type C57BL/6 mouse model, levels of Fas-mediated apoptosis increase early in infection in a nonrandom fashion, with parietal and chief cells preferentially affected (81, 85). Subsequently, proliferation is increased and homeostasis is maintained at the cost of increased cell turnover. As infection persists and metaplastic and dysplastic populations arise, cells become resistant to Fas-mediated apoptosis (86, 87) and may use the Fas pathway instead for proliferative signaling (80). Nitric oxide, while usually discussed in the context of DNA damage and mutagenesis, can directly influence mitochondrial pathways of apoptosis (88), and potentially plays a role in multiple levels of cell signal transduction during infection. Additionally, bacterial factors may directly induce apoptosis. VacA has been reported to insert directly into the membrane of mitochondria, inducing cytochrome c release and apoptosis (89) (Fig. 1.3). This is described in more details in **CHAPTER III**.

6. Cell cycle regulation.

Several bacterial factors have been directly implicated in altering apoptotic and proliferative signaling. For instance, *Helicobacter* infection alters expression of the cell cycle regulatory protein p27, which appears to confer an apoptosis- resistant phenotype to cells in culture and alters the cellular response to chemotherapeutic agents (90-92).

7. Cell - cell cross talk and signal disruption.

Parietal cell loss is temporally associated with atrophy, mucous cell metaplasia and initiation of dysplastic changes (93), suggesting a cause and effect relationship. In addition to production of acid, the parietal cell regulates key differentiation decisions in the fundic oxyntic glands with ablation of parietal cells associated with the appearance and expansion of undifferentiated cell types. Deregulated proliferation of poorly differentiated precursor cells is often associated with the appearance of a mucus neck cell lineage expressing trefoil factor 2 (TFF2) or spasmolytic polypeptide (SP). TFF2 is felt to have a physiological role in cytoprotection and maintenance of mucosal integrity and repair (94, 95), because it is usually expressed at the edges of healing ulcers, with downregulation of expression once restitution and healing are complete. Continued expression, as is seen in mucous cell metaplasia, is associated with dysplasia and progression to cancer; however, causality has not been shown. Another effect of parietal cell loss is hypochlorhydria, hypergastrinemia, and bacterial overgrowth, which may contribute independently to tissue damage and abnormal cell signaling.

8. Summary.

H. pylori remains one of the most common chronic bacterial infections in the world. Fifteen to twenty percent of infected patients will develop gastric or duodenal ulcer disease and up to 1% will develop gastric adenocarcinoma (30). Unfortunately, symptoms and disease are poorly correlated and many cases of gastric cancer are detected

late in disease progression, when they are incurable. Evidence to date clearly stresses that a vital factor in the induction of *Helicobacter*-related disease is the host immune response. Bacterial factors most responsible for initiating and continuing the immune response continue to be evaluated. Our understanding of the bacterial interactions with components of the innate immune response continues to evolve. Additionally, we are expanding our understanding regarding the components of the adaptive immune response responsible for disease initiation and progression. A multitude of host and environmental factors, including interaction with other infectious agents and dietary factors, affect the immune response to *Helicobacter* organisms and may impact disease presentation over the life of the host. The presence of sophisticated analytical tools such as human and mouse genome sequence data, mouse models of infection and gastric cancer and the complete genome and evolving functional analysis of the *H. pylori* genome will prove extremely valuable as we address still unanswered questions on the pathogenesis of *H. pylori* induced gastric cancer.

CHAPTER II. MATERIALS AND METHODS

1. Mice.

The University of Massachusetts Institutional Animal Care and Use Committee approved all animal work. Six- to 8-week-old male C57BL/6 mice which were viral antibody (Ab) free, parasite free, and bacterial pathogen free, inclusive of *Helicobacter* species, were purchased from Jackson Laboratories (Bar Harbor, ME) and housed in microisolator cages, fed standard chow, and allowed free access to water.

T-bet (Th1-specific T box transcription factor) KO (knockout) mice in a C57BL/6 background were certified free of *H. felis*, *Helicobacter hepaticus*, and *Helicobacter bilis*, were purchased from The Jackson Laboratory and crossed with C57BL/6 mice and heterozygous mice mated to produce T-bet KO and C57BL/6 wild type littermates as controls. Male and female mice were genotyped according to company protocol before initiation of any experiments and again at euthanasia. An equal number of male and female mice were used for each study.

2. Helicobacter and Toxoplasma infections.

Helicobacter felis (strain 49179 from the American Type Culture Collection, Manassas, VA) and *Helicobacter pylori* Sydney strain (a kind gift from Timothy Wang MD) were grown on Trypticase soy agar with 5% sheep blood under microaerophilic conditions at 37° for 4 days. Bacteria were harvested and the number of CFU (colony forming units) determined as previously described (85). Mice were restrained by hand

and gavaged with *Helicobacter felis* or *H. pylori* (1 X 10^7 CFU suspended in 400 µl total volume) via a 20-gauge feeding tube (Popper and Sons, New Hyde Park, N.Y.) three times at 2-day intervals.

The 76K strain of *T. gondii* was maintained by continuous oral passage of cysts in female C57L/J mice. After 2 months of infection, mice were euthanized, brains removed, and cysts counted. To induce *T. gondii* infection, 100 *T. gondii* cysts suspended in 250 µl PBS were given by oral gavage. For the effects of chronic *Helicobacter* infection, mice were infected with *H. felis* 20 wk before infection with *T. gondii*. For acute *Helicobacter* infection, mice were first infected with *T. gondii*, and on days 5, 7, and 9 post infection (to coincide with the maximum IFN-γ levels induced by *T. gondii*), mice were infected with *H. felis*. Control mice were infected with *T. gondii* alone, *H. felis* alone, or mock infected with vehicle alone. Mice were euthanized before predetermined time points if they became moribund or showed evidence of severe dehydration or distress.

3. Bone marrow transplantation.

Bone marrow was isolated from the femurs and tibias of 6-8 week old C57BL/*6JGtrosa26* (ROSA26) or C57BL/6-TgN [ACTbEGFP] (Green Fluorescent Protein) mice. Total bone marrow was washed, triturated using a 20-gauge needle and passed through a 40µm nylon mesh cell strainer (Becton Dickson, Franklin Lakes, NJ) to produce a single cell suspension in PBS. Recipient C57BL/6J mice were irradiated with 900 rads from a cesium 137-gamma cell irradiator, reconstituted with 3 x 10^6 donor

marrow cells via a single tail vein injection, and used for experiments after 4 weeks of recovery. The overall level of engraftment was 60-80% as assessed by analysis of beta-galactosidase or GFP in peripheral leukocytes. Non-transplanted C57BL/6 mice and C57BL/6 transplanted with C57BL/6 marrow from liter mates served as controls for all injury models. C57BL/6 mice transplanted with marrow from ROSA26 or GFP transgenic mice without further intervention served as controls for baseline engraftment. All mice received a single 1 mg/kg BrdU intraperitoneal injection prior to euthanasia.

4. Acute stomach alcohol injury.

Mice were gavaged with a dose of 200µl of 50% ethanol in normal saline (NaCl 0.9%, pH 7.2), as per Andrade et al. (96). Each mouse was given one dose a day for four successive days, then sacrificed at day 5. This corresponds to a dose of 5gm of alcohol/kg of mouse weight. Each mouse weighed 30gm.

5. Bone marrow culture.

Total marrow was collected as described above into PBS containing penicillin/streptomycin 1% and 5% fetal calf serum. Red Blood Cells were lysed. Bone marrow was depleted of differentiated cells using an antibody cocktail of TER, YW25.12.7, MAC-1, GR-1, LYT-2, L3T4, B220 and separated using Dynal™ magnetic bead. Depleted bone marrow was stained with Rhodamine and Hoechst dye and a lineage

depleted, Rhodamine dull, Hoechst dull ($lin^{-}Rho^{dull}Ho^{dull}$) population isolated as the HSC population (76) using the Cytomation MoFlo (Dakocytomation, Carpenteria CA). Culturing whole marrow in complete medium isolated mesenchymal stem cells (DMEM, FBS 10%, L-glutamine 1%, penicillin/streptomycin 1%, sodium pyruvate 1%, and nonessential amino acids 1%) for 3 days, discarding non-adherent cells, and retaining adherent cells as MSC (mesenchymal stem cells). HSC or MSC (2000 cells) were plated into the upper well of a 0.4μm pore 6.5 mm Transwell culture dish with polycarbonate membrane (Corning Costar Corporation, Cambridge MA). The bottom dish contained either complete medium or complete medium containing gastric mucosa (see above). Cells were cultured at 37°, 5% CO2 for 24 or 48 hours.

6. RT-PCR of bone marrow cells.

RNA was isolated using RNeasy Protect Mini kit, and reverse transcribed using equivalent amounts of RNA using Omniscript Reverse Transcriptase and PCR with HotStarTaq Master Mix kit as per manufacturers instructions (Qiagen, Valencia, CA, USA). Primer sequences were as follows: Keratin19 (KRT1-19) 189bp, Annealing temp 59°C, forward primer 5′- CCGCGGTGGAAGTTTTAGTGG-3′, reverse primer 5′-GGTCCGGGTCCCTGCTTCTGGTA-3′. CD45 277bp, annealing temperature 51°C, forward primer 5′-GCACAC CAAAAGAAAAGGCTAATA-3′, reverse primer; 5′-GGAATCCCCAAATCTGTCTGC-3′. GAPDH 210bp, Annealing temperature 57°C, forward primer 5’GACATCAAGAAGGTGGTGAAGC-3′, reverse primer 5′-GTCCACCACCCTGTTGCTGTAG-3′. TFF2 annealing temperature 58°, forward primer

5′-CTGGTAGAGGGCGAGAAA-3′, reverse primer 5′- AGAAACACCAGGGCACTT-3′ all for 45 cycles and product resolved on a 2% agarose gel with ethidium bromide.

7. Culture and characterization of culture adapted Mesenchymal stem cells (MSC).

Total marrow was isolated as described above. Once confluent, cells were cultured with DMEM/20% FCS. At each passage, cultures were split as follows: one plate was held in culture at confluence for detection of foci, one plate was split into the next passage culture, and the remainder of the plates was frozen. Surface expression of CD44 and CD45 (BD PharMingen) was assessed by fluorescence-activated cell sorting (FACS) analysis. Assessment of lineage specific markers, cytokeratin, vimentin, desmin, CD34, CD31, S100, and smooth muscle actin (SMA)(BD PharMingen), was determined by immunohistochemistry or reverse transcription-PCR (RT-PCR). Selected MSCs were stably transfected with the plasmid pDS-Red-monomer-hyg-C1, red fluorescent protein (RFP) expression was verified by RT-PCR and FACS, and single-cell clones were isolated.

8. Mesenchymal stem cells injection in mice.

10^6 RFP labeled MSC were injected in mice by a single tail vein injection on day 3 after the 3rd dose of alcohol was given. Prior to injection, the cells were washed in PBS,

strained through a 40μm strainer and suspended in 500μl of PBS.

9. Necropsy and histology.

Mice were euthanized, the stomach removed, opened longitudinally along the greater curvature, and gently washed with PBS (phosphate buffered saline). Strips of gastric tissue along the lesser curvature from the squamocolumnar junction through the pylorus were taken, fixed in 10% neutral buffered formalin for 4 h, processed by standard methods, embedded in paraffin, cut into 5-μm sections, stained with H&E, and examined for inflammation and architectural distortion.

Sections were scored in a blinded fashion as follows: *Inflammation* 0: Normal; 1: Small multifocal leukocyte accumulations in mucosa; 2: Coalescing mucosal inflammation; early submucosal extension; 3: Coalescing mucosal inflammation with prominent multifocal submucosal extension ± follicle formation; 4: Severe diffuse inflammation of mucosa, submucosa, with or without deeper layers. *Hyperplasia* 0: Normal; 1: one and one-half times normal thickness; 2: two times normal thickness with mitotic figures one-third way up to surface; 3: three times normal thickness with mitotic figures half way up to surface; 4: four times normal thickness or greater with mitotic figures greater than half way up to the surface. *Parietal cell loss and mucous cell hypertrophy and metaplasia* 0: no substantial alterations; 1: <5% alteration; 2: 25–50% alteration; 3: 50–75% alteration; 4: >75% alteration.

For enumeration of parietal cells, five high-power fields in each H&E

(hematoxylin & eosin) - stained section were examined; beginning at the junction between the forestomach and fundus, with two well-oriented gastric glands counted per field for a total of 10 glands per mouse (97) and reported as the average number of parietal cells per gland ± 1 SD.

10. Preparation of tissue for immunohistochemistry.

Mice were euthanized by CO2 inhalation; gastric tissue was removed, washed, sectioned as described above and fixed in 10% neutral-buffered formalin, 100% ethanol, or Prefer (Anatech, Ltd., Battle Creek, MI), followed by standard histological processing. Sections were stained using routine H&E. For immunohistochemistry, sections were deparafinized and hydrated. Permeabilixation with PBST (0.2% Tween) was done at room temperature for 10 min, followed by antigen retrival with urea (1M) at 100°C in microwave. Next, H2O2 quench (H2O2-3% in methanol) for 20 min at room temperature. After a three times wash with PBST (phosphate buffered saline Tween), avidin/biotin block was applied (Vector Laboratories, Burlingame, CA). Serum blocking applied for 1 h at room temperature with 5% solution of the host of secondary antibody. Primary antibody was applied overnight at 4°C in prior determined concentrations. The next day, after a quick wash the secondary antibody was applied at room temperature for 1 hour. If the secondary antibody was biotinilated, an ABC mixture was applied as per manufacturer's instructions (Vector Laboratories, Burlingame, CA), followed by DAB developing. Sections were labeled using antibodies directed against BrdU (Zymed, San

Francisco, CA), bacterial betagalactosidase (Promega, Madison, WI), GFP (Abcam, Cambridge, UK), RFP (Rockland, Gilbertsville, PA).

For immunostaining using mouse antibodies on mouse tissue the ARK kit (DAKO, Carpinetera, CA) was employed. For dual fluorescence immunohistochemistry, cell phenotype markers were indirectly labeled with streptavidin-fluorescein using antibodies directed towards pan-cytokeratin (AE1/AE3; DAKO) or CD45 (leukocyte common antigen, Ly-5, BD Biosciences, San Diego, CA) followed by a biotin block and serial application of anti-beta-galactosidase antibody labeled indirectly with streptavidin-Cy3. Sections were mounted with anti-fade Vectashield with DAPI (Vector Laboratories, Burlingame, CA), fields viewed and captured with the Zeiss Axiopath system (Thornwood, NY), and serial 3-color images were overlaid using Photoshop 6.0 (Adobe Systems, San Jose, CA).

11. Direct fluorescence on stomach frozen sections.

Tissue was first fixed in 4% paraformaldehyde for 4h at room temperature, then embedded in OTC compound (SAKURA, Torrance, USA) and immediately frozen. 5μm frozen sections were then cut, fixed in methanol/acetic acid (3:1 ration) for 10 min. After a quick air dry the slides were mounted with DAPI solution and examined for fluorescence under a microscope.

12. Co-localization of Y-chromosome and cytokeratin in paraffin tissue sections.

Collected 5μm paraffin tissue section were coated on slides, and then dried overnight at 37°C. Slides were the dewaxed in xylene and rehydrated in graded alcohols to water. After incubating the slides in sodium thiocyanate (1M) for 10 min at 80°C, they were washed in water and digested in pepsin solution (50 mg in 100 ml of NaCl at pH of 1.5 of HCl) at 37°C for 10 min. It was followed by a glycine solution (2 mg/ml) treatment at room temperature for 5 min to neutralize the pepsin effect. Slides were then fixed in 4% paraformaldehyde for 2 min at room temperature. After washing in PBS three times of 15 min each, the tissue was dehydrated in graded alcohols, then air dry. The slides were then aged at 65°C for 1 hour. After the StarFish^{TM} Y chromosome-FITC conjugated probes (purchased from Cambio, UK) were applied on the slides, they were denatured for 10 min at 65°C, then incubated at 37°C for overnight. The next day, slides were washed and exposed to the FITC amplification kit as per manufacturer's protocol (Cambio, UK). For cytokeratin detection, tissue was then exposed to anti-pankeratin PE conjugated antibody (clone C11, Abcam, Cambridge) at a concentration of 1/50 for 20 min at 37°C. After a quick wash, slides were mounted with DAPI (4',6-diamidino-2-phenylindole) antifade and cover slips applied; then immediately viewed with a fluorescent microscope (Olympus, Japan).

13. Determination of enzyme (X-gal) activity.

After intracardiac perfusion (4% paraformaldehyde, 2% glutaraldehyde in 0.1 M Sorensen's phosphate buffer (pH 7.4) with 2 mM MgCl2 and 5 mM EGTA), stomachs were removed, opened along the greater curvature, washed, and linear longitudinal sections from the squamocolumnar junction through the pylorus collected. Tissue was embedded in OCT compound (SAKURA Torrance USA), snap frozen, and sectioned on a cryostat for enzyme histochemistry. Frozen sections (10μm) were washed in Sorensen's buffer containing 0.01 % sodium deoxycholate and 0.02 % Nonidet P-40 and incubated for 4 hours at 37°C in a 0.1 % X-gal solution (4 % 4-chloro-5-bromo-3- indolyl-D galactopyranoside (X-gal) dissolved in dimethylformamide, 5 mM K3Fe (CN) 6.5 mM $K_4Fe(CN)6.6H_2O$ in 0.1 M Sorensen's phosphate buffer) and counterstained with nuclear fast red. Additional sections had nuclear fast red omitted, and cells were instead labeled using antibodies directed against TFF2 (a kind gift from N. A. Wright).

14. Laser capture microdissection of beta-galactosidase positive glands.

Frozen sections were prepared and stained with X-gal as outlined above. Individual gastric cells within positive glands, individual lymphocytes within an area of submucosal infiltrate or negative glands (control) were captured using the Laser Capture Microscope PixCell II, (Arcturus Engineering Inc., Mountain View, CA, U.S.A). Twenty to 30 cells were captured for each area of interest. DNA was extracted using the PicoPure DNA extraction kit (Arcturus Engineering Inc., Mountain View, CA, USA) and GAPDH (210

bp) (2) or 140 bp of the LacZ/NEO fusion gene sequence amplified as follows: Annealing temperature 56°, forward primer 5′-CGGGCTGCAGCCAATATGGGATCG- 3′, reverse primer 5′-GCCGGAACACGGCGGCAT*CAGA*GC-3′ Using HotStarTaq Master Mix (Qiagen, Valencia, CA, USA). Primer sequence for LacZ/NEO was a kind gift from D.K. Kotton.

15. Mouse gastric mucosal cell culture.

The entire stomach was removed from C57BL/6 mice, opened along the greater curvature and washed extensively in sterile PBS, 20% FCS (fetal calf serum), supplemented with penicillin/streptomycin and amphothericin. The fundic mucosa was scraped free from the serosa using a sterile scalpel, minced, manually disaggregated using a pipette, and the cell suspension equally divided into the bottom wells of the Transwell™ culture plate. Medium was changed after 24 hours to remove dead cells.

16. Single stomach cell preparation for FACS analysis and FISH (fluorescent in situ hybridization).

Stomachs were removed, opened and extensively washed. A small longitudinal section from the squamocolumnar junction through the antrum was processed as outlined above with standard H&E, anti-beta galactosidase or cytokeratin IHC. The fundic mucosa was gently scraped free from the serosa, minced and digested for 2 hours in 1mM EDTA, 2% BSA and 0.1% pronase in PBS at 37° and filtered through a 40 μm nylon mesh

strainer. For determination of DNA content, cells were fixed in 70% ethanol for 48 hours, stained with PI, and DNA content determined by FACS. For X and Y chromosome determination, single cell preparations from stomachs of sex-mismatched, GFP marrow transplanted mice were prepared as detailed above. Cells were washed twice in PBS on ice, resuspended in 0.5 ml of staining buffer (2% FBS, 0.01% sodium azide in PBS) and stained with anti-CD45-PE Ab (BD Pharmingen) according to manufacturer protocol. FACS sorted cells, which were GFP+ and CD45-, GFP+ and CD45+, GFP- and CD45+ and GFP- and CD45- were collected. An aliquot of each was prepared and analyzed for DNA content as described above and analyzed using FloJo software. For cytokeratin immunocytochemistry, an aliquot of cells were spun onto slides (Thermo Shandon) at a speed of 500 rpm for 5 minutes at a density of 5,000 cells per slide, fixed in 4% paraformaldehyde pH of 7.4 for 1 hour and incubated with the primary Ab for AE1/AE2 keratin complex (DAKO Corporation, Carpinteria, CA) at a dilution of 1/50 using the Animal Research kit (DAKO corporation, Carpinteria, CA) followed by incubation with diaminobenzidine/hydrogen peroxidase as chromogen-substrate and counterstained with hematoxylin. X- and Y-chromosomes were detected using the Dual Color Detection kit (ID Labs) according to the manufacturer's protocol (DAPI for nuclear staining, FITC for Y chromosomes and Texas Red for X chromosomes) and immediately viewed with a fluorescent microscope (Olympus, Japan).

17. Cytokeratin staining of stomach single cells after FACS sorting.

Fluorescence-activated cell sorter (FACS)-sorted cells were spun onto slides (Thermo

Shandon, Pittsburgh, PA) at a speed of 500 rpm for 5 min at a density of 5,000 cells per slide, fixed in 4% paraformaldehyde, pH 7.4, for 1 h, and incubated with the primary Ab for AE1/AE2 keratin complex (DAKO Corporation, Carpinteria, CA) at a dilution of 1/50 using the Animal Research kit (DAKO Corporation, Carpinteria, CA), followed by incubation with diaminobenzidine/hydrogen peroxidase as a chromogen substrate and counterstaining with hematoxylin.

18. Serum IFN-γ determination in mice.

Blood was collected before euthanasia and immediately spun; aliquots of serum frozen at -80°C. IFN-γ ELISA detection kit (Pierce Endogen, Rockford, IL) was used according to manufacture's protocol. Samples were run in duplicate, compared with a standard curve generated with known values, and reported as the mean ±SD for each group.

19. ELISA for IgG1 and IgG2a Abs against H. felis.

The 96-well plates were coated overnight at 4°C with 100 µl of a 10 µg/ml *H. felis* protein in carbonate buffer (pH 9.6) as previously described (65). The blocked washed plates were incubated for 1.5 h at room temperature with diluted serum samples in triplicate. Biotinylated monoclonal secondary Abs produced by clones G1-6.5 and R19-15 (BD Pharmingen, San Diego, CA) for detecting mouse IgG1 and IgG2a, respectively,

were used. Incubation with streptavidin peroxidase (Sigma-Aldrich, St. Louis, MO) was followed by ABTS substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD) for color development. Absorbance at wavelength 405 nm was recorded on an ELISA plate reader (DynatechMR7000; Dynatech Laboratories, Chantilly, VA) and reported at a sample dilution of 1/100.

20. ELISA for anti-T. gondii IgG-total, and IgG1 and IgG2a isotypes.

Sera from mice were tested for *T. gondii*-specific IgG and IgG subtypes as previously described (98). Briefly, 96 microwell plates were coated with *T. gondii* tachyzoites (5 X 104 parasites per well), dried overnight then blocked with PBS-BSA 4% at 37°C for 1 h. Serial dilutions of the sera (1/200 to 1/512,000 for total IgG and 1/1000 to 1/1,024,000 for isotype analysis) were incubated for 1 h at 37°C followed by incubation with anti-mouse IgG conjugated to alkaline phosphatase (1/1000 dilution; Sigma- Aldrich) or anti-mouse IgG1 or IgG2a conjugated with HRP (1/8000 dilution; Southern Biotechnology Associates, Birmingham, AL) for 1 h at 37°C and detected by *p*-nitrophenyl phosphate or tetramethylbenzidine substrate (Sigma-Aldrich), respectively. OD was measured at 450 nm with an ELISA plate reader (Bio-Rad, Hercules, CA). The Ab titer for each sample was calculated as described by (99) and expressed as the reciprocal of the highest dilution for which the absorbance was 2.5 times greater than the absorbance of control sera at the same dilution. Results are given as the means of log2 titer ± SD.

21. Quantitative analysis of H. felis colonization.

A 2 mm X 2 mm piece of gastric mucosa taken at the fundus/antral border was snap frozen at the time of necropsy. All samples were processed together as follows: DNA was extracted using High Pure PCR Template Preparation kit (Roche Molecular Biochemicals, Indianapolis, IN) according to manufacture's protocol. The 2 µl of extracted DNA was used for RealTime PCR (SmartCycler; Cepheid, Sunnyvale, CA) using QuantiTect SYBR Green PCR kit (Qiagen, Valencia, CA). Standards were made by sequential 10-fold dilutions of purified *H. felis* DNA producing a range from 500,000 to 5 copies per reaction. This is based on the premise that 2 Fg of *H. felis* chromosomal DNA is equivalent to 1 copy of the *H. felis* genome. Each sample was analyzed in triplicate. Primers sequences for a 225 fragment of the *flaB* gene were as follows: 5'-TTCG ATTGGTCCTACAGGCT*CAGA*-3' and 5'TTCTTGTTGATGACATTGACCA ACGCA- 3'. Annealing temperature was 55°C.

22. Quantification of splenic T. gondii tachyzoite DNA.

One-half of each spleen was snap frozen at the time of necropsy and all samples processed together. A 5 µl of genomic DNA was used for Real- Time PCR (SmartCycler; Cepheid) using QuantiTect SYBR Green PCR kit (Qiagen) according to manufacturers

directions. Results were analyzed using relative quantification with $2^{-\Delta\Delta CT}$ method (100) using GAPDH as the internal control. Primers amplifying a 183 bp fragment of the *T. gondii B1* gene were as follows: 5'-CATTCTTGTGCTGCCTCCTCTCAT-3', 5'-AGCGGCAGCGTCTCTTCCTCTTT-3', annealing temperature at 57°C. The other half of the spleen was fixed in 10% buffered formalin and processed as previously described. *T. gondii* tachyzoites were enumerated in spleen sections stained with H&E.

23. Spleen cell assays.

Spleen cells were isolated using Lymphocyte Separation Medium-M (Cellgro). For anti-CD3 stimulation, High Protein binding plates (Corning) were coated with anti-CD3 Ab at 400 ng/ml. Spleen cells were plated at 1 X 10^6 per well together with anti-CD28 at 100 ng/ml (BD Pharmingen) and incubated for 18 h. Cytokine levels were measured in culture supernatants by ELISA. Statistical analysis was performed using Student's *t* test (JMP Software). For FACS analysis, spleen cells were harvested and RBCs were lysed with Tris-ammonium chloride. Cells were incubated with allophycocyanin- or PE-labeled Abs specific for FasL, CD3, CD4, CD8, CD11b, CD19, and CD69 (BD Pharmingen) and analyzed using a FACS scan analyzer (BD Biosciences).

24. RT-PCR of stomach sorted cells.

Populations ($MHCII^+$ Fas Ag^+ $CD45^-$, $MHCII^+$ Fas Ag^- $CD45^-$, and mouse stomach)

were subjected to reverse transcription-PCR (RT-PCR) for the following genes: Fas Ag, MHCII, TFF2, gastric intrinsic factor (GIF), hydrogen potassium ATPase (HK ATPase), MUC5, MUC6, and 18S rRNA. RNA was isolated using the RNeasy kit (QIAGEN, Valencia, CA), purified, and DNase treated using a DNA-free RNA kit (Zymo Research, Orange, CA). Reverse transcription was done with the Omniscript RT kit (QIAGEN, Valencia, CA) using the manufacturer's protocol. PCR was done with the HotStarTag Master Mix kit (QIAGEN, Valencia, CA) using the manufacturer's protocol, and the PCR products were run on an agarose gel (2%). RT-PCR was carried out in a programmed thermal Eppendorf Mastercycler (Eppendorf AG, Hamburg, Germany) using primer sequences and conditions listed in Table 1.

Table 1. Mouse primers.

Primer name	Primer sequences (Forward and Reverse)	Product size (bp)	Annealing Temp (°C)
Fas Ag	AAGGGAAGGAGTACATGGACAAGA GAGGCGCAGCGAACACAG	184	55
MHC class II	GACATTGGCCAGTACACATTTGAA TTGGGGAACACAGTCGCTTGAG	230	55
TFF2	GCTTCCCGGGCATCACCAG AAACACCAGGGCACTTCAAA	229	56
GIF	TTGCCCAAATTCTCCCTTCCTT CCCCTCTCAGCTGGTTGTTTATGG	171	55
HK ATPase	CCCCCAATGGCACCTTCAGTCTCC TTCGTAGGGGTCATGGGGGTTGTT	182	59
MUC5	ACGGGGATGGCCACTTTGTTACCT TCTCCGCTTGGCCCTTGCTCTAC	253	59
MUC6	ACTGGCCAGCCTGTCCGAAACT GGGGCCATAAACCATACCATTGAG	255	60
18S rRNA	AAAATAGCCTTCGCCATCACTG GGCTGTACTTCCCATCCTTCAC	217	56
IFN-γ	CATGGCTGTTTCTGGCTGTTACTG GTTGCTGATGGCCTGATTGTCTTT	226	54.5
IL-12	CTGCCTGCCCCCA*CAGA*AGA GCG*CAGA*GTCTCGCCATTATG	208	57
IL-1β	CAGGATGAGGACATGAGCACC CTCTG*CAGA*CTCAAACTCCAC	446	60
IL-4	ATCGGCATTTTGAACGAGGTCA CATCGAAAAGCCCGAAAGAGTCT	221	56
IL-10	CCTAGAGCTGCGGACTGCCTTCA CAGCCGCATCCTGAGGGTCTTC	247	58
GAPDH	GACATCAAGAAGGTGGTGAAGC GTCCACCACCCTGTTGCT GTAG	210	57
IL-5	GGCTTCCTGTCCCTACTCATAAAA AGCCTTCCATTGCCCACTCT	252	55
IL-13	ATGGCGCTCTGGGTGACTGC ATTTTGGTATCGGGGAGGCTGGAG	326	60.1
CXCR4	GACCGCCTTTACCCCGATAGC ACCCCCAAAAGGATGAAGGAGTC	248	57.9
β actin	CCTAAGGCCAACCGTGAAAAGATG GTCCCGGCCAGCCAGGTCCAG	219	59.7
SDF-1	GAGAGCCACATCGC*CAGA*GC GGATCCACTTTAATTTCGGGTCAA	123	60

25. Real-time PCR for gastric mucosal cytokines.

Total RNA was extracted from the fundus of the stomach using RNAeasy kit (Qiagen). A 5 μg of total RNA was DNase treated using RNase-Free DNase set (Qiagen) and reverse transcribed using OmniScript RT kit (Qiagen). A total of 2 μl of cDNA was used for RealTime PCR (SmartCycler; Cepheid) using QuantiTect SYBR Green PCR kit (Qiagen) and results analyzed using Relative Quantification with the $2^{-\Delta\Delta CT}$ method (100). GAPDH or β-actin was used as the internal control. Primers were designed using DNASTAR software (Madison, WI). Primer sequences are listed in Table 1.

26. Cell culture of RGM1 cells.

Rat gastric mucosal cells (RGM-1 cell line; RIKEN cell bank, Tsukuba Science City, Japan) were cultured in Dulbecco's modified Eagle medium with 20% fetal bovine serum, 1% L-glutamine, and 1% penicillin-streptomycin (Invitrogen, Grand Island, NY) at 37°C (with 5% CO2) and grown to 50 to 70% confluence. Cell lines were cultured as described above with 100ng/ml rat gamma interferon (IFN-γ) (PeproTech, Rocky Hill, NJ) for 48 h, with 0.5 μg/ml cytochalasin D (CytoD) (Calbiochem, San Diego, CA) for 18 h, and/or with 12.5, 25, or 50 ng/ml human recombinant Fas L for times ranging from 30 min to 48 h (50% lethal dose = 50 ng/ml; Alexis Corporation, San Diego, CA). The parent cell line and cell lines expressing Fas Ag with or without MHCII were exposed to cytochalasin D prior to exposure to Fas L. The dose used in this study (0.5 μg/ml)

inhibits actin polymerization without being cytotoxic, as assessed by analytical cytology analysis (101).

27. Characterization of RGM-1 cells.

Total RNA was extracted from RGM1 and MHCII-Fas Ag cells and rat stomach (as a positive control) by using TRIzol (Invitrogen, Life Technologies, Carlsbad, CA) according to the manufacturer's directions. Two micrograms of RNA was purified, DNase treated, and reverse transcribed as previously described. Primer sequences for rat TFF2, GIF, TFF2, HK ATPase β subunit, MUC, MUC1, MUC5, MUC6, and 18S rRNA are given in Table 2.

Table 2. Rat primers.

Primer name	Primer sequences (Forward and Reverse)	Product size (bp)	Annealing Temp (°C)
TFF2	CTGGCGGAAGGCGAGAAAC ATGCCCGGGTATCCACAAT	230	57
GIF	ATTGCC*CAGA*TTCTCCCTTCCT CCCCCTCAGCTGGTTGTGTTTATG	171	55
HK ATPase	TCACGGCGGACATGCTA*CAGA*AT TCCGGGGCTTTTGGGGGTCATC	174	58
MUC	CACCCCCATCTCCACCACCATTAC AGCCCCTCTGAGACATTACACTG	240	56
MUC1	CCAGTGCCGCCGAAAGAGC CAGCCGGGTTGGTGTAAGAGA	191	58
MUC5	CCAAGGGCCTCCACCAACAC TCCAGGCCTGAGCACACACC	201	57
MUC6	ACACCTGTGGGCCCCTCCTACTTG ACTGTGGGCCTTGTGGGTGTTGAC	209	59
18S rRNA	TCCCCGAGAAGTTTCAGCACATCC CTTCCCATCCTTCACGTCCTTCTG	269	59
MHC class II	TCCCTGCGGCGGCTTGAG TCTGACGCTTGTGACGGATGAAAA	406	60
Ii (invariant chain)	CCCGATGCGCATGGCTACTCC CCCGCGGCTCTTGGTGTGA	487	60
CIITA (major histocompatibility class II transactivator)	TGCCCACGAAACACAGGAACC GACGGGGCCCAATGCAAACTCTA	466	60
GAPDH	TCTTCACCACCATGGAGAA ACTGTGGTCATGAGTCCTT	231	60

Periodic acid-Schiff (PAS) staining was performed on cytospun cells by using a PAS staining system from Sigma (St. Louis, MO) according to the manufacturer's protocol. Alcian blue staining was performed using alcian blue solution (alcian blue 8GX [Sigma, St. Louis, MO] at 1 g in 100ml 3% acetic acid, pH 2.5) for 30 min, followed by a tap water wash for 2 min and counterstaining with Nuclear Fast Red (DAKO, Carpinteria, CA) for 5 min. Slides were dehydrated and mounted with Permount (Fisher Scientific, Fair Lawn, NJ).

28. RGM-1 cells as a model for MHCII signaling in gastric mucosa.

Total RNA was extracted from RGM1 cells exposed to 100 ng/ml rat IFN-γ (PeproTech, Rocky Hill, NJ) or control medium and was reverse transcribed and analyzed as previously described, using primers for MHCII RT1B-beta chain, MHCII transactivator (CIITA), and GAPDH (glyceraldehyde-3-phosphate dehydrogenase). Control reactions were done without the addition of reverse transcriptase.

29. Fas Ag- and MHCII - expressing clones.

RGM-1 cells expressing high levels of surface Fas Ag, MHCII complex alone, both Fas Ag and MHCII complex, or empty vector were made as follows. Total RNA was isolated from mouse thymus and reverse transcribed, and specific sequences were amplified using the following primers: I-A alpha sense, 5'-

CTAGCTAGCACCATGCCGCGCAG*CAGA*GCT-3'; I-A alpha antisense, 5'-CCGGAATTCTCATAAAGGCCCTGGGTGTC-3'; I-A beta sense, 5'-TGCTCTAGAACCATGGCTCTG*CAGA*TCCCCA- 3'; and I-A beta antisense, 5'-ATAAGAATGCGGCCGCTCACTGCAGGAGCCCTG-3'. After sequencing (University of Massachusetts Nucleic Acid Facility; ABI Prism model 377 version 3.4.1, ABI200), the 771-bp MHCII αchain was inserted into the NheI/EcoRI site of multiple cloning site A and the 791-bp MHCII β chain was inserted into the XbaI/NotI site of multiple cloning site B of the pIRES cloning vector (Clontech, Palo Alto, CA), and stable clones were selected in neomycin. The 1-kb Fas Ag fragment was cloned using the sense primer 5'-GAAGATCTG*CAGA*CATGCTGTGGATCTGGGCTGTC-3' and antisense primer 5'-CTCGAATTCTCACTC*CAGA*CATTGTCCTTCATTTTC- 3', sequenced, and inserted into the BglII/EcoRI site of pMSCV-puro (BD Clontech), and stable clones were selected in puromycin. Membrane surface receptor expression was confirmed by FACS analysis (anti-CD95-FITC or MHCII [I-Akα-FITC and I-Akβ-PE]; PharMingen, San Diego, CA). Expression of MHCII and Fas Ag was analyzed as a percentage of positive cells over background staining. Before these studies, expression of Fas Ag or MHCII on CD45- depleted, *H. felis*-infected (4 to 8 weeks) mouse gastric mucosa was determined by FACS, and populations were classified as high- or low-expressing populations based on surface receptor abundance. The average receptor expression level in the high-abundance population was quantitated relative to GAPDH and 18S rRNA. We then chose our high-Fas-Ag-expressing cell lines and our dual Fas Ag/MHCII cell lines based on the numbers derived from these studies. A cell line was considered a high expressor if it

expressed 1.5 to 2.0 times the average level of Fas Ag, or of Fas Ag and MHCII, of the mouse gastric mucosal cells. Three to five representative clones of each group were used to confirm experiments.

30. RGM1 cell growth assays.

For proliferation, 1 X 10^4 cells per well were incubated in 96-well plates with or without 12.5 ng/ml Fas L for 24 h. The plates were harvested and [^{3}H] thymidine incorporation measured as counts per minute. For apoptosis assessment, 1 X 10^4 cells were seeded into each well of a six-well plate and incubated with or without Fas L at 25 ng/ml for 4 h. Cells were visualized, stained with annexin V and propidium iodide by using an annexin V-FITC kit (Oncogene, Boston, MA) according to the manufacturer's protocol, and analyzed via flow cytometry. Cells that were annexin V positive and PI (propidium iodine) negative or annexin V positive and PI positive were determined to be apoptotic. Experiments were repeated three times.

31. Caspase 8 activity assay.

Cells (1 X 10^6) were stimulated for 1 h with Fas L at 25 ng/ml. Stimulated and control cells were collected and analyzed using a Caspase-8 Fluorometric Activity Assay kit (Oncogene, Boston, MA) according to the manufacturer's directions. Caspase 8 activity was measured using a fluorescent plate reader (Perkin-Elmer Life Sciences, Boston, MA) at an excitation wavelength of 400 nm and an emission wavelength of 510 nm.

Comparisons were made in relative fluorescence units. Experiments were repeated three times.

32. DISC immunoprecipitation and analysis.

Cells were grown to 70 to 80% confluence and then stimulated with human recombinant FLAG-tagged-Fas L (Alexis Biochemicals) (50 ng/ml) or control vehicle for 30 min. Cells were harvested, washed in cold buffer, pelleted, and lysed in ice-cold lysis buffer (30 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride) with 2 μg/ml small peptide inhibitors (pepstatin, chymostatin, antipain, and leupeptin; Sigma, St. Louis, Missouri) for 30 min. Control cells had FLAG-tagged Fas ligand added after lysis. Cell debris was pelleted by centrifugation for 10 min at 13,000 X *g*. FLAG-Fas L-CD95-DISC complexes were immunoprecipitated with an affinity column and anti-FLAG M1 affinity gel (Sigma, St. Louis, MO) according to the manufacture's protocol. The multimeric status of Fas Ag/CD95 was determined by fractionating immunoprecipitated complexes under non reducing conditions on a 4 to 20% gradient gel (Gradipore Ltd., French Forest, Australia) at 4°C. Proteins were transferred to a polyvinylidene difluoride membrane (Amersham Biosciences Corp, Piscataway, NJ) and blocked overnight in 5% nonfat dry milk in 0.1% Tris-buffered saline–Tween 20. Anti-CD95 mouse monoclonal Ab at a 1:2,000 dilution (BD PharMingen, San Diego, CA) was used as the primary Ab, and donkey anti-mouse horseradish peroxidase-conjugated Ab (Santa Cruz Biotechnology Inc., Santa Cruz, CA)

at a 1:3,000 dilution was used as the secondary Ab. To verify components of DISCs, aliquots were run on reducing gels and blotted using anti-Fas Ag monoclonal antibody at a 1:2,000 dilution (BD PharMingen, San Diego, CA) and rabbit anti-mouse caspase 8 polyclonal antibody at a 1:1,000 dilution (BD PharMingen, San Diego, CA), which detects both noncleaved (inactive) and cleaved forms of caspase 8. Detection was done using the ECL Plus System kit (Amersham Biosciences Corp., Piscataway, NJ) according to the manufacturer's directions.

33. CXCR4 knock down in mesenchymal stem cells.

shCXCR4-GFP construct (clones 1, 2, 3) was purchased from GeneCopoeia (Rockville, MD). The cells were transfected with this constructs using a letiviral system. 293FT cells were used for virus production using Virapower (Invitrogen, Carlsbad, CA) as per manufacture's instruction manual, under collaboration with Dr. John Mordes. Cells were infected with clones 1, 2 and 3 in succession, using 50µl of viral culture per well of a 6 well plate at a 70% cell confluence. The infection efficiency was checked by GFP fluorescence.

34. cAMP (cyclic adenosine monophosphate) ELISA assay.

50,000 cells per well were seeded in a 96 well plate, precoated with anti-cAMP antibodies, purchased from Applied Biosystems (Bedford, MA). After letting the cells to

grow in culture overnight, they were stimulated with SDF-1 at 100ng/ml concentration for 10 min at 37°C. Cell media was then aspirated and the cells were lysed in a buffer provided in the kit. The remaining protocol was followed as per manufacturer's instructions. The results were read by a plate luminometer at an emission of 470nm (Perkin-Elmer, Waltham, MA). The results were plotted against the standards, provided in the kit.

35. Transwell cell migration assay.

Transwell plates with an 8μm pore size inserts were purchased from Corning (Lowell, MA). Mesenchymal cells were seeded in the well insert at a 10,000-cells/100μl of 0.1% fetal calf serum concentration media. After a 6-hour incubation, the bottom well media was changed to new media with or without 100ng/ml of SDF-1 and incubated for overnight. The next day, the inserts and the media were discarded. The cells that migrated through the Transwell were fixed with methanol/acetic acid (3:1 ratio) for 10 min, and then stained with Giemsa solution (1/20 concentration) for 10 min. After a wash with PBS, the plate was air-dried and the cells were counted under a microscope. The results of migrated cells were plotted as a ratio of its nonstimulated control group (given a value of 1).

36. Western blotting on cultured cell lines.

Cells were grown at 80% confluence overnight, seeded in equal numbers. Cells were then lysed by adding 1xSDS sample buffer (500µl per a 100mm plate). Sonication was done for 10-15 seconds to shear DNA and reduce sample viscosity. After a 5 min heating done at 100°C, cell lysates were centrifuged for min and the supernatant was loaded on a SDS-PAGE gel. Transfer was done on PFDF (Biorad, Hercules, CA) membrane for 65 min at 110 volts. Blocking was done with 5% nonfat milk at room temperature for 1 hour. Primary antibody at appropriate concentrations (CXCR4 – 1/250 (Abcam, UK); β-actin HRP conjugated, 1/1000 (Santa Cruz, CA)) was incubated overnight at 4°C. The next day, secondary antibody was incubated for 1 hour at room temperature. Detection was done using SuperSignal (Pierce Endogen, Rockford, IL).

37. Statistical analysis.

Survival after *T. gondii*/*H. felis* infection is shown using the Kaplan-Meier curve. Data for serum IFN-γ, *T. gondii* gene expression, tachyzoite quantitation, and *H. felis* copy number are reported as the mean ± 1 SD. Results for *T. gondii* Ab response are given as the mean of log2 titer ±SD. *H. felis* IgG1 and IgG2a are reported as a mean ± 1 SE and compared using the Student *t* test. A value of $p < 0.05$ was considered statistically significant for differences between groups. Pathology data are compared using the Mann-Whitney analysis of nonparametric data and considered significant at a value of $p < 0.05$.

Statistical analysis of real-time RT-PCR was done using REST 2009 software (Technical University Munich, Germany).

CHAPTER III. MAJOR HISTOCOMPATIBILITY COMPLEX CLASS II INHIBITS FAS ANTIGEN-MEDIATED GASTRIC MUCOSAL CELL APOPTOSIS THROUGH ACTIN-DEPENDENT INHIBITION OF RECEPTOR AGGREGATION

Introduction

Escape from normal apoptotic control is thought to be essential for the development of cancer. During *Helicobacter pylori* infection, the leading cause of gastric cancer, activation of the Fas antigen (Fas Ag) apoptotic pathway is responsible for early atrophy and tissue loss. As disease progresses, metaplastic and dysplastic glands arise, which express Fas Ag, but are resistant to apoptosis and are believed to be the precursor cells for adenocarcinoma. The C57BL/6 mouse model of *Helicobacter* infection is a powerful tool with which to study the initiation and progression of gastric cancer. Infection in the C57BL/6 mouse recapitulates human disease, progressing from atrophy and metaplasia through dysplasia to intraepithelial neoplasia with invasion through the muscularis mucosae (102). Using this mouse model to understand the mechanism(s) by which gastric mucosal cells acquires a transformed phenotype is critical to our efforts to prevent and treat gastric cancer. Initiation of cancer requires that cells escape from normal apoptotic controls (103). The extension of cell viability through inhibition of apoptosis is a hallmark of human tumors and appears necessary for cells to acquire a transformed phenotype (103). While inhibition of apoptosis per se is not carcinogenic, failure to remove genetically damaged cells which otherwise would have been eliminated allows unchecked accumulation of potentially transforming mutations. *Helicobacter* has been shown to induce apoptosis both directly and indirectly in gastric mucosal cells (104-111). One apoptotic pathway is the Fas antigen (Fas Ag) signal cascade. Under normal, noninflamed conditions, Fas Ag is expressed at minimal levels throughout the gastric mucosa (112). However, with *Helicobacter* infection there is a marked upregulation of

mucosal Fas Ag receptor expression (81, 84), and an increase in Fas Ag expression in both mouse experimental systems and in vitro tissue culture systems results in an increase in apoptotic signaling (80, 83-85). Fas signaling is crucial for gastric mucosal alterations under inflammatory conditions (81, 83, 113-115). As the time of infection increases, a subset of cells within the gastric mucosa appear which are resistant to Fas mediated apoptosis as evidenced by continued survival in the face of persistent Fas Ag expression and available ligand. The Fas Ag-expressing population of cells increases as architectural alterations progress through metaplasia to dysplasia to cancer (87, 112). The mechanism of Fas-apoptosis resistance is not known, but has been suggested to include expression of FAP-1 (Fas associated phosphatase-1), an inhibitor of Fas-mediated apoptosis (86) and FLIP (FLICE inhibitory protein), a dominant- negative caspase 8 (87). In general, Fas-mediated cell death is controlled by an abundance of independent mechanisms allowing cells to integrate a variety of intracellular and extracellular signals to respond in a highly flexible manner towards Fas stimulation. Regulation of the Fas pathway in gastric cells is complex and, at present, incompletely defined.

Fas Ag is a cell surface receptor. When Fas ligand (Fas L) binds to Fas Ag, receptors aggregate and recruit adaptor proteins, forming the death-inducing signaling complex (DISC) (consisting of Fas L, Fas Ag, FADD (Fas-Associated protein with Death Domain) and FLICE [procaspase 8]). Once the DISC is formed, a caspase cascade is activated directly (type I signaling) or activated using a mitochondrial amplification loop (type II signaling). Both pathways converge at the level of caspase 3, resulting in apoptosis. There are multiple levels at which this cascade can be regulated, including regulation by parallel signaling pathways and receptor-receptor interactions.

Major histocompatibility complex class II (MHCII) molecules are heterodimeric cell surface glycoproteins whose expression is critical for the development of CD4 T cells and the ability to mount an adaptive immune response. These molecules are expressed on professional antigen-presenting cells, such as B cells, macrophages, and dendritic cells, which function in the uptake, processing, and presentation of antigen to CD4 T cells. In addition, these molecules are constitutively expressed on epithelial cells of the thymus, macrophages, Langerhans cells of the skin (116), and Kupffer cells in the liver (117). Under permissive conditions, MHCII expression can be induced in a variety of cell types, including cells of the gastrointestinal tract (104, 118).

During *Helicobacter* infection, gastric mucosal cells upregulate surface MHCII expression and costimulatory molecules (119-121) and have the ability to weakly present antigens (119, 122). Additionally, there is evidence that *Helicobacter pylori* may bind the gastric cell via the MHCII complex and regulate apoptotic responses (120). We show that during *Helicobacter* infection there is a small but distinct population of gastric mucosal cells, which coexpress Fas Ag and MHCII, setting up a scenario where these receptors may interact, akin to what we see in immune cells. Our examination of cells in culture demonstrates that MHCII prevents Fas receptor aggregation at the level of the actin cytoskeleton and renders Fas-expressing gastric mucosal cells resistant to Fas L-mediated apoptosis. These findings support that MHCII coexpression may be one of the mechanisms by which gastric mucosal cells escape Fas-mediated apoptosis.

Results

1. RGM-1 cells resemble immature gastric mucus cells.

Metaplastic and dysplastic cells express both MHCII and Fas Ag and are Fas apoptosis resistant. In order to test the interaction of these two receptors in vitro, we used a cell line derived from mucus cells that resembles cells early in the metaplasia-dysplasia sequence. RGM-1 cells are cytokeratin positive but do not express lineage-specific markers such as GIF (expressed in chief cells), hydrogen potassium ATPase (expressed in parietal cells), MUC5 or MUC6 (expressed in neck and surface epithelial cells), or TFF2 (expressed in metaplastic cells) (Fig. 3.1A) and do not stain with alcian blue, pH 2.5 (indicative of intestinal type mucin) (data not shown). RGM-1 cells are weakly PAS positive (Fig. 3.1B to G), indicating glycogen stores.

2. A population of gastric mucosal cells expresses both Fas Ag and MHCII receptors under conditions of inflammation.

During *Helicobacter* infection and inflammation, Fas Ag (81, 83, 85, 113-115) and MHCII (119-121, 123) expression on the gastric mucosal cell surface is increased. Representative histological sections from sham-infected (Fig. 3.2A), *H. felis*-infected (Fig. 3.2B), and *H. pylori*-infected (Fig. 3.2C) mice at 6 months show preserved normal architecture in the sham-infected mouse, with a paucity of inflammatory cells and no metaplasia or dysplasia (Fig. 3.2A). At 6 months (Fig. 3.2B and C), the chronically

FIGURE 3.1 RGM-1 CELLS RESEMBLE IMMATURE MUCUS CELLS.

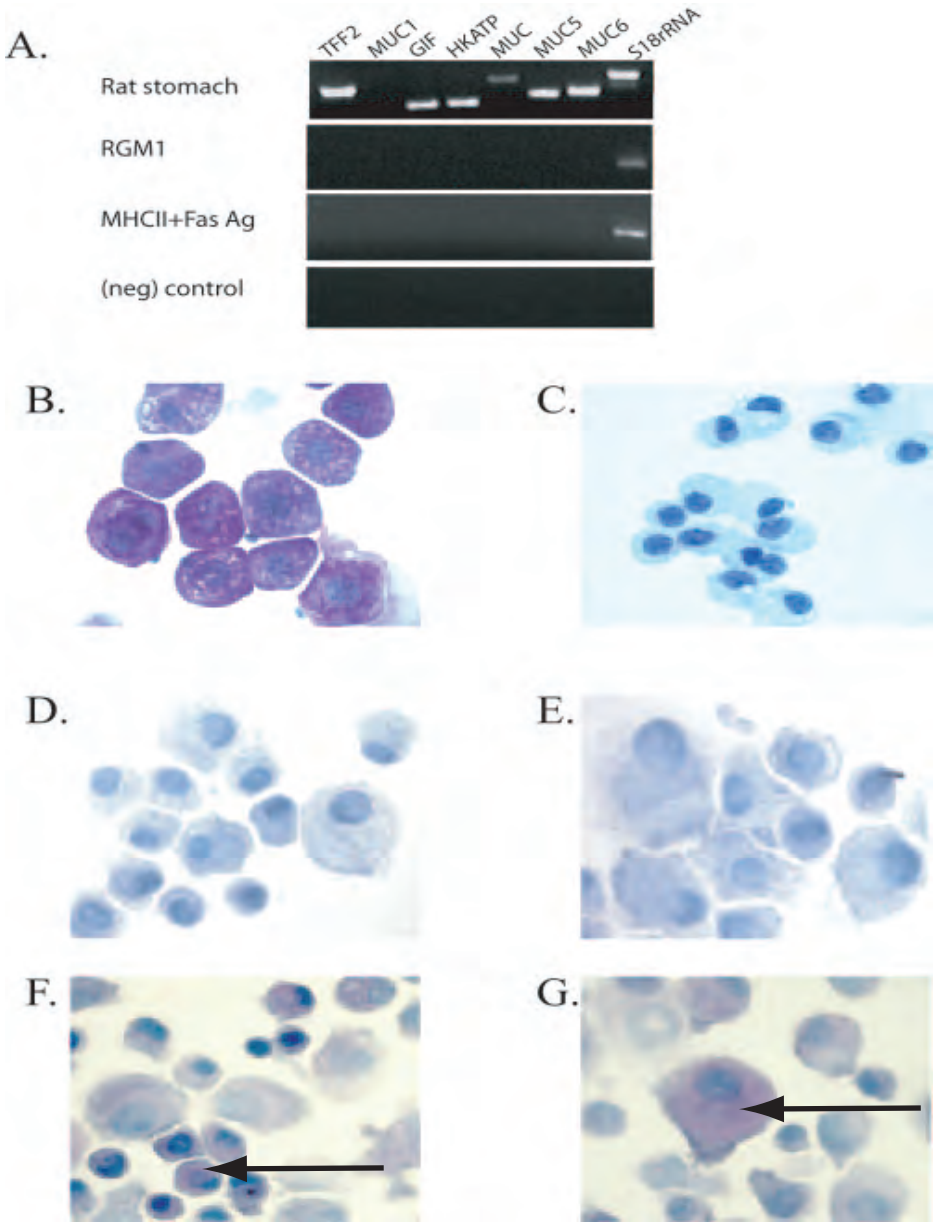


FIGURE 3.1 RGM-1 CELLS RESEMBLE IMMATURE MUCUS CELLS.

(A) RT-PCR for a panel of gastric mucosa-expressed genes in RGM-1 and RGM-1 MHCII/Fas Ag cell lines. Total rat stomach was used as a positive control (top row). RGM-1 cells without reverse transcriptase were used as a negative control (bottom row). RGM1 and RGM-1 MHCII/Fas Ag cells do not express any of the gastric mucosa genes showing that they are immature cells. **(B and C)** PAS-hematoxylin staining of primary hepatocyte culture is positive (purple staining of cytoplasm) (positive control) **(B)** and primary lymphocyte culture is negative for PAS staining (negative control) **(C)**. **(D and E)** Hematoxylin staining of RGM-1 **(D)** and MHCII/Fas Ag **(E)** cells. **(F and G)** PAS - hematoxylin staining of RGM-1 **(F)** and MHCII/Fas Ag **(G)** cells. Both cell lines are positive for PAS staining (arrows).

FIGURE 3.2 HISTOLOGICAL CHANGES DUE TO *HELICOBACTER* INFECTION.

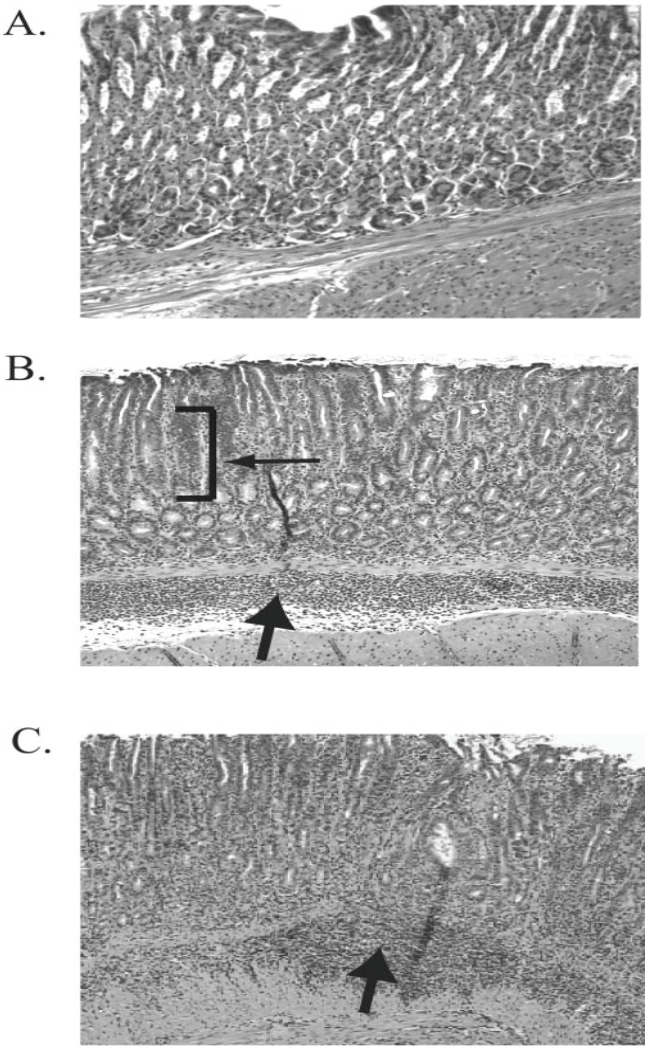


FIGURE 3.2 HISTOLOGICAL CHANGES DUE TO *HELICOBACTER* INFECTION.

Six- to eight-week-old male C57BL/6 mice were infected with control medium (sham infected), *H. felis*, or *H. pylori* and euthanatized at 6 months. The gastric mucosa of sham-infected mice **(A)** had normal architecture, with preservation of parietal cells and a paucity of inflammatory cells. The gastric mucosa of mice infected with *H. felis* **(B)** or *H. pylori* **(C)** had antralization of glands (thin arrow), loss of parietal cells, and chronic inflammatory infiltrate in the submucosa (thick arrows) and above the muscularis mucosa, between glands. Hematoxylin and eosin staining was used; magnification, X40.

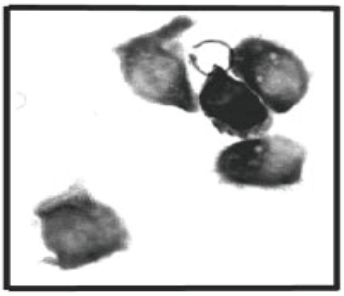
infected mice have developed moderate submucosal plasma-lymphocytic infiltrates, with lymphocytes invading the mucosal layer and intercalating between glandular units. There is a decrease in the number of parietal and chief cells, antralization of glands and early metaplasia. There was no evidence of dysplasia at this early time point. The findings were similar for both the *H. felis*- and *H. pylori*-infected groups; however, the range of mucosal alterations was broader with *H. pylori* infection. *H. felis* infection, which showed less mouse-to-mouse variation, was used for the remainder of the experiments. To determine both the percentage of cells expressing Fas Ag and MHCII and whether expression occurred within the same gastric mucosal cell, we examined single-cell preparations of the gastric mucosa that had been depleted of immune cells (CD45+) by FACS. Under control conditions, MHCII is expressed on a small minority of gastric mucosal cells, and only 0.12% of cells coexpressed Fas Ag and MHCII. In contrast, mice infected with *Helicobacter felis* expressed both MHCII and Fas Ag on the surface of greater than 6% of the gastric mucosal cells isolated (representative data from one experiment are shown in Fig. 3.3A). Cytokeratin staining confirmed that these dual-receptor-expressing cells were epithelial cells (Fig. 3.3B and C). We further determined the nature of these dual-receptor expressing cells. We analyzed whole gastric mucosa (containing all gastric cell lineages and inflammatory cells) and two populations of CD45-negative gastric cells, a double-positive population expressing both Fas Ag and MHCII and a double negative population that expressed neither Fas Ag nor MHCII, for the expression of lineage specific markers. Fas Ag and MHCII are coexpressed on cells expressing a wide range of lineage markers, suggesting that this double-receptor-expressing population is a heterogeneous mixture of parietal, chief, mucous, and

FIGURE 3.3 FAS AG AND MHCII ARE COEXPRESSED ON GASTRIC EPITHELIAL CELLS DURING *HELICOBACTER* INFECTION.

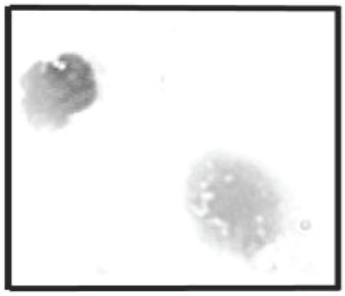
A.

	% of cells expressing			Total number of cells		
	Fas Ag	MHCII	Both	Fas Ag	MHCII	Both
Control	21.36%	0.03%	0.12%	1.7×10^6	2,350	9,403
H. felis infected	53.90%	0.07%	6.14%	4.2×10^6	5,484	4.8×10^5

B.



C.



D.

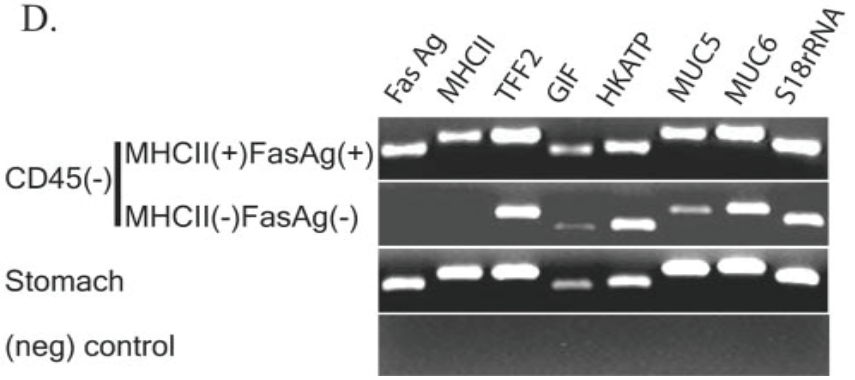


FIGURE 3.3 FAS AG AND MHCII ARE COEXPRESSED ON GASTRIC EPITHELIAL CELLS DURING *HELICOBACTER* INFECTION.

(A) Single-cell preparations from control or *H. felis*-infected mice were stained with anti-CD45-Cy5 conjugated, anti-Fas Ag-PE conjugated, and anti-MHCII-FITC conjugated antibodies and FACS (fluorescent acquisition cell sorting) sorted into CD45 (-) populations expressing Fas Ag, MHCII, or Fas Ag/MHCII. Results of one representative experiment are shown. The experiment was performed three times with similar results. Cells from mice infected with *H. pylori* gave a similar pattern of expression, and results are not shown. **(B** and **C)** Cytokeratin staining of CD45(-)MHCII/Fas Ag(+) **(B)** and CD45(+) **(C)** populations confirms that the CD45 (-) MHCII/Fas Ag(+) are of an epithelial nature. **(D)** RT-PCR for expression of a panel of lineage specific genes. The CD45(-) population was sorted into MHCII/Fas Ag-expressing (top row) and non-MHCII/Fas Ag-expressing cells (second row) populations. cDNA prepared from total mouse stomach was used for controls. Third row, positive control; fourth row, without reverse transcriptase, negative control.

metaplastic cells (Fig. 3.3D).

3. Gastric mucosal cells express MHCII in response to IFN-γ.

In order to study MHCII/Fas Ag signal interactions, we turned to a tissue culture system. Rat gastric mucosal (RGM-1) cells in culture do not express MHCII or costimulatory molecules under normal culture conditions (Fig. 3.4A, lane 1). Upon exposure to IFN-γ, MHCII, invariant chain (Ii) and CIITA expression are markedly increased at both the mRNA (Fig. 3.4A, lane 2) and protein (Fig. 3.4B) levels. Control experiments were done without reverse transcriptase enzyme to exclude genomic DNA sequence amplification (Fig. 3.4A, lanes 3 and 4). RGM-1 cells cultured with IFN-γ for 24 to 72 h elongate and develop an increased number of cytoplasmic projections (Fig. 3.4C and D). RGM-1 cells express surface Fas Ag after exposure to the inflammatory cytokines IL-1β, TNF-α, and IFN-γ (85), similar to what is seen in vivo, further supporting the use of this cell line as a model for MHCII/Fas interaction in gastric mucosal cells.

4. Gastric cell lines expressing MHCII have altered morphology and growth characteristics.

In order to address potential interactions between MHCII and Fas Ag in gastric mucosal cells, while avoiding potentially confounding effects of exogenous IFN-γ, we

FIGURE 3.4 IFN-GAMMA REGULATES MHCII EXPRESSION IN GASTRIC MUCOSAL CELLS.

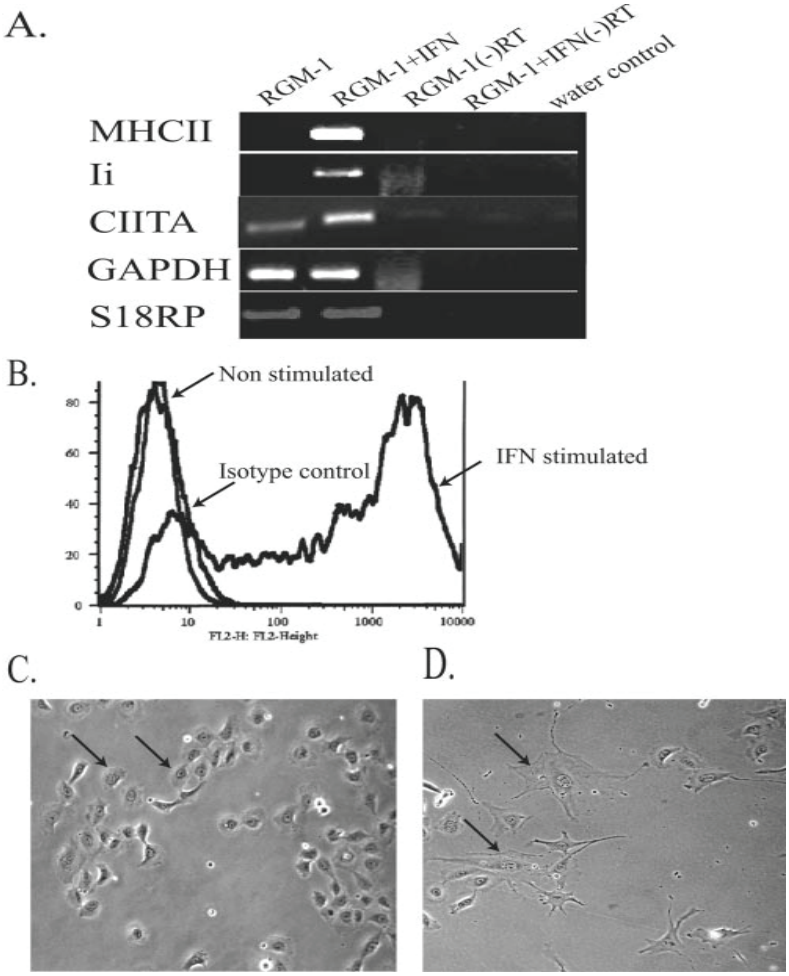


FIGURE 3.4 IFN-γ REGULATES MHCII EXPRESSION IN GASTRIC MUCOSAL CELLS.

Rat gastric mucosal cells (RGM-1) were cultured in the absence or presence of 100 ng/ml rat IFN-γ for 24 to 72 h. **(A)** RT-PCR for MHCII, Ii (invariant chain), and CIITA (class II major histocompatibility complex transactivator) expression after 24 h in culture. GAPDH and S18 ribosomal message were used to standardize the blots. **(B)** Surface MHCII expression was confirmed by FACS analysis. **(C)** Cells grown for 72 h in control medium have a compact hexagonal shape (arrows). **(D)** Cells grown with 100 ng/ml IFN-γ for 72 h are elongated with stellate projections (arrows).

established cell lines stably expressing the MHCII complex and the Fas Ag receptor. FACS analysis confirmed surface expression of both the MHCII alpha and beta chains (Fig. 3.5A and B) and Fas Ag (Fig. 3.5C). Clones, which expressed high physiological levels (see Materials and Methods) of both receptors were chosen for further study. In culture, RGM-1 cells have a hexagonal shape and rarely show cell projections (Fig. 3.5C). RGM-1 cells transfected with empty vector or those expressing high levels of Fas Ag alone did not differ in size, shape, or growth characteristics from the control cell line (data not shown). Interestingly, cell lines expressing MHCII alone or MHCII/Fas Ag (Fig. 3.5D) had a flattened, fusiform, spindle-shaped phenotype with long cytoplasmic "hummingbird" projections and were phenotypically indistinguishable from RGM-1 cells exposed to IFN-γ (Fig. 3.5D). There was no increase in spontaneous apoptosis or cell death in any of the cell lines.

5. Gastric mucosal cells expressing MHCII are resistant to Fas-mediated cell signaling.

In culture, RGM-1 cells express negligible Fas Ag surface receptor; have a low level of spontaneous apoptosis, and have a very small increase in apoptosis in response to Fas L (Fig. 3.6A and B). RGM-1 cells expressing MHCII on the cell surface also have a low baseline level of apoptosis, which is not increased, with the addition of Fas L (Fig. 3.6C and D). In sharp contrast, RGM-1 cells expressing abundant Fas Ag on the cell surface rapidly undergo apoptosis in response to the addition of 25.0 ng/ml Fas L, with

FIGURE 3.5 MORPHOLOGICAL ALTERATIONS OF GASTRIC MUCOSAL CELLS EXPRESSING BOTH MHCII AND FAS AG.

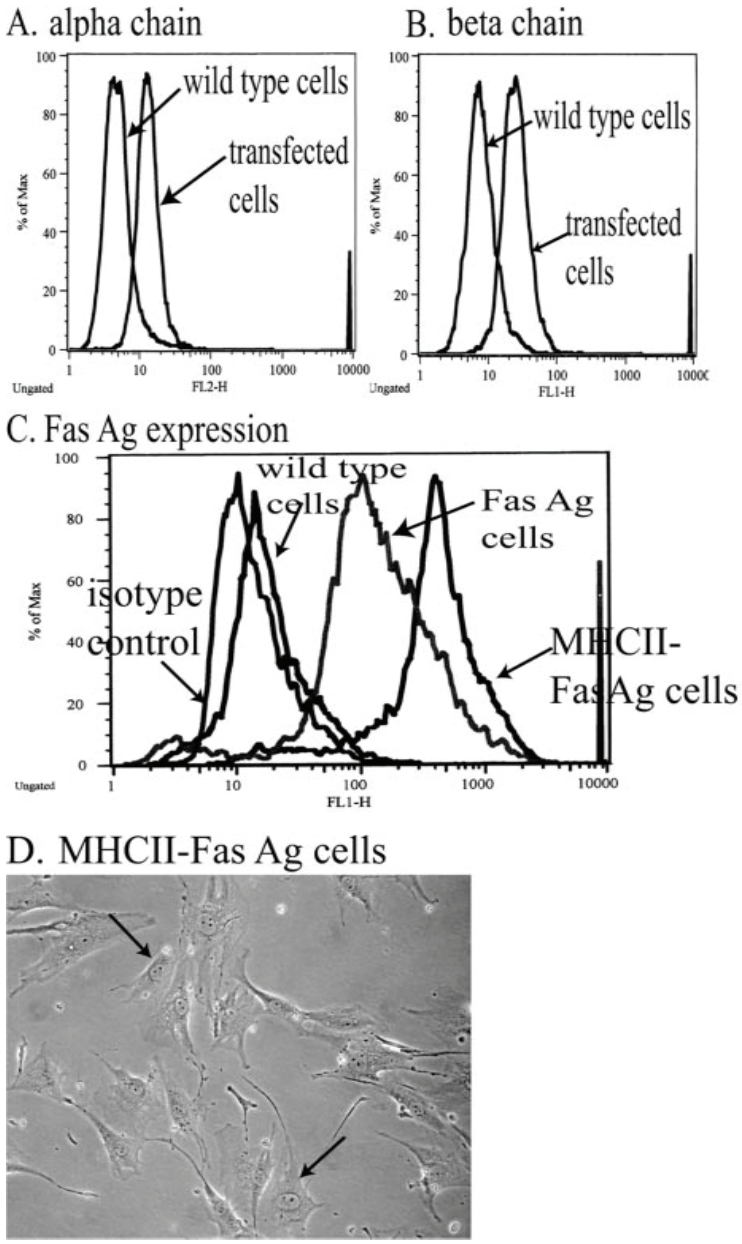


FIGURE 3.5 MORPHOLOGICAL ALTERATIONS OF GASTRIC MUCOSAL CELLS EXPRESSING BOTH MHCII AND FAS AG.

RGM-1 cells were transfected with appropriate expression vectors. **(A** to **C)** Expression of alpha chain **(A)**, beta chain **(B)**, and Fas Ag **(C)** surface receptors was confirmed by FACS analysis. **(D)** Cellular morphology after 72 h in culture. Arrows show long stellate projections.

FIGURE 3.6 GASTRIC MUCOSAL CELLS EXPRESSING MHCII AND FAS AG ARE RESISTANT TO FAS L INDUCED APOPTOSIS.

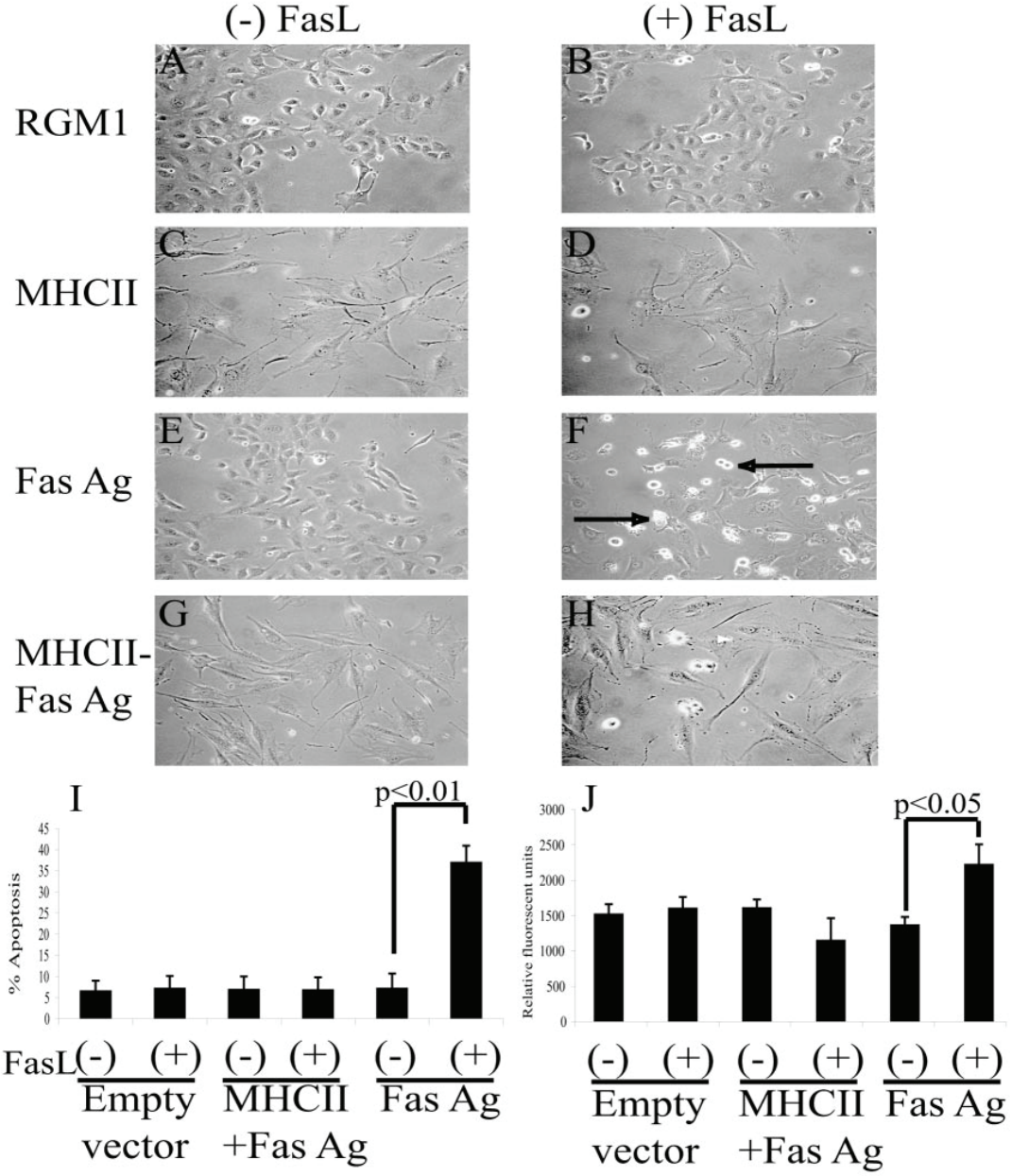


FIGURE 3.6. GASTRIC MUCOSAL CELLS EXPRESSING MHCII AND FAS AG ARE RESISTANT TO FAS L INDUCED APOPTOSIS.

Cells were exposed to control medium or medium containing 25 ng/ml Fas L for 4 h. **(A** and **B)** RGM-1 cells have a low basal level of apoptosis and do not undergo morphological changes of apoptosis when exposed to ligand. (**C** and **D**) Cells expressing MHCII do not undergo apoptosis under control conditions or upon exposure to Fas L. **(E** and **F)** Cells expressing Fas Ag do not undergo spontaneous apoptosis but show rounding, detachment, and blebbing (arrows) upon addition of ligand. **(G** and **H)** Cells with a higher level of Fas Ag expression as than in panels (**E)** and **(F)** but also expressing MHCII do not undergo spontaneous apoptosis and do not increase apoptosis in response to ligand. **(I)** Levels of apoptosis were quantitated using annexin V staining in cell lines with or without 25 ng/ml Fas ligand for 4 h as indicated. **(J)** Cells were grown under the same culture conditions as for panel **(I)** for 1 h, and caspase 8 activity was measured using the Caspase-8 Fluorometric Activity Assay kit. All experiments were repeated three times. Results in panels (**I)** and (**J)** are averages from three experiments ±1 standard deviation.

38% ± 5% of cells being apoptotic at 4 h (Fig. 3.6E and F) and greater than 98% being apoptotic at 24 h (data not shown). Strikingly, cells expressing both MHCII and Fas Ag are completely resistant to Fas L-induced apoptosis (Fig. 3.6G and H). Dual-receptor-expressing cells did not undergo apoptosis in response to 25.0 ng/ml Fas L for 4 or 24 h (Fig. 3.6G and H; summarized in 3.6I) and showed no increase in caspase 8 activity (Fig. 3.6J), suggesting that the block to signaling was upstream of caspase activation. Gastric mucosal cells can use the Fas Ag pathway for proliferative signaling at low receptor stimulation (due to low receptor abundance or low ligand level) (80, 85) and for both proliferative and apoptotic signaling at high receptor stimulation (80, 85, 124). We next tested whether Fas proliferative signaling was intact in cells, which coexpress MHCII and Fas Ag. Consistent with our previous reports, RGM-1 cells expressing low-abundance Fas receptor exposed to low-level Fas L (6.25 or 12.5 ng/ml) significantly increased proliferation (p <0.01) compared with cells exposed to control culture conditions. Cells expressing both MHCII and high levels of surface Fas Ag had no growth response to ligand. Dual-receptor-ex-pressing cells had very low baseline levels of proliferation as measured by [^{3}H] thymidine incorporation, which did not change with the addition of 6.25 to 25 ng/ml Fas L (Fig. 3.7), suggesting that all Fas-mediated growth signaling was interrupted in these cells.

6. MHCII inhibits Fas signaling by preventing receptor aggregation.

In order to determine the mechanism of Fas resistance, we first determined the

FIGURE 3.7 MHCII COEXPRESSION INHIBITS FAS-MEDIATED PROLIFERATIVE SIGNALING.

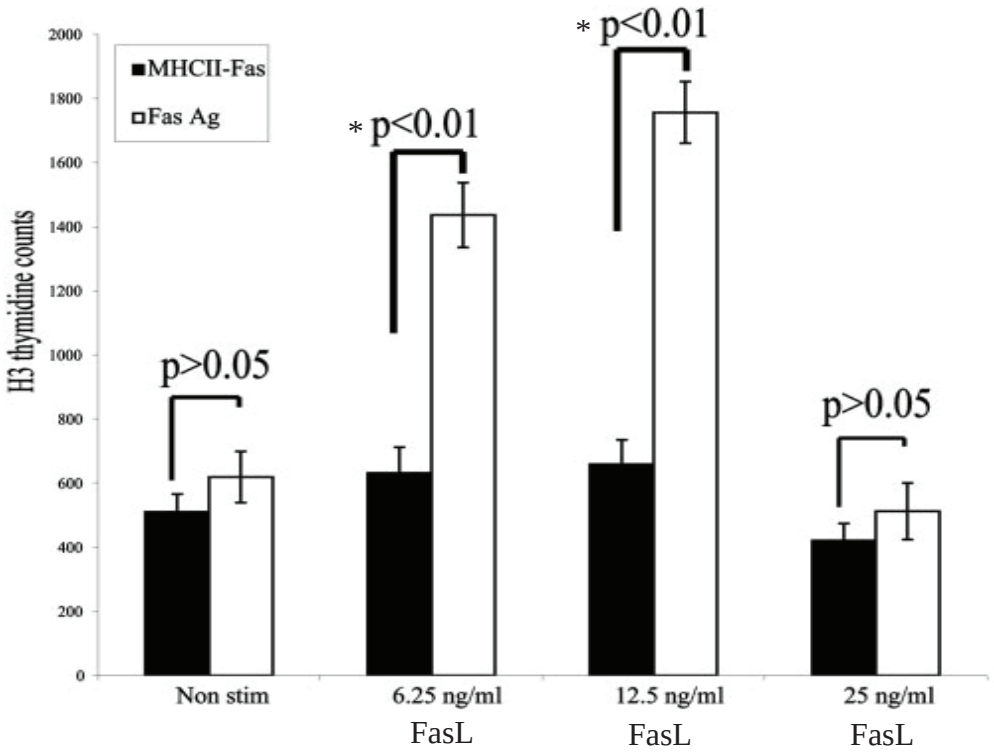


FIGURE 3.7 MHCII COEXPRESSION INHIBITS FAS-MEDIATED PROLIFERATIVE SIGNALING.

RGM-1 cells expressing MHCII and Fas Ag or Fas Ag alone were cultured for 24 h in control medium or medium containing 6.25 to 25 ng/ml Fas ligand (as indicated), and proliferation was measured by $^{[3H]}$ Thymidine incorporation. Experiments were repeated three times, and results represent the averages ±1 standard deviation.

point within the pathway where regulation likely occurred. Caspase 8 is not activated in dual-receptor-expressing cells; therefore, MHCII interferes with Fas signaling at or above the level of caspase 8 activation (Fig. 3.6J). Levels of the inhibitor FLIP were unchanged between cell lines (data not shown). Because of the alteration in cell morphology with MHCII, we addressed whether receptor aggregation was impaired. FLAG-tagged Fas L was used to immunoprecipitate DISCs (Fas L, Fas Ag, FADD, and FLICE) from Fas Ag expressing and Fas Ag/MHCII-expressing cell lines. After the addition of ligand to Fas Ag-expressing cells (Fig. 3.8A, lane 3), only signal-competent 250-kDa multimeric complexes were isolated (Fig. 3.8A, top gel). These bands were confirmed to be specific for DISCs by running an aliquot on a denaturing gel and blotting with Fas Ag (Fig. 3.8A, middle gel), which produced only a single 48-kDa band for each sample, or caspase 8 (Fig. 3.8A, bottom gel). Strikingly, in cells expressing both Fas Ag and MHCII, a significant portion of receptors remained as 75-kDa monomeric complexes and failed to aggregate (Fig. 3.8A, lane 1); this failure to aggregate could not be overcome with either longer incubation times or higher levels of ligand (data not shown). Scant uncleaved caspase 8 (50 to 55 kDa) was recovered from these DISCs (Fig. 3.8A, third gel, lane 1).

Inhibition of actin polymerization by CytoD completely restored receptor clustering, as only aggregated receptor complexes were recovered (Fig. 3.6A, lane 2). In addition, abundant caspase 8 was recovered in these DISCs and existed as both uncleaved (50- to 55-kDa) and active (41- to 43-kDa) forms, comparable to that which was detected in Fas Ag-only expressing cells (lane 3). In the presence of CytoD, MHCII/Fas Ag-expressing cells lost their stellate cytoplasmic projections and assumed a more hexagonal shape, similar to wild-type control cells (data not shown). With the addition of ligand, caspase 8

FIGURE 3.8 MHCII INHIBITS FAS AG SIGNALING THROUGH AN ACTIN-DEPENDENT INHIBITION OF RECEPTOR AGGREGATION.

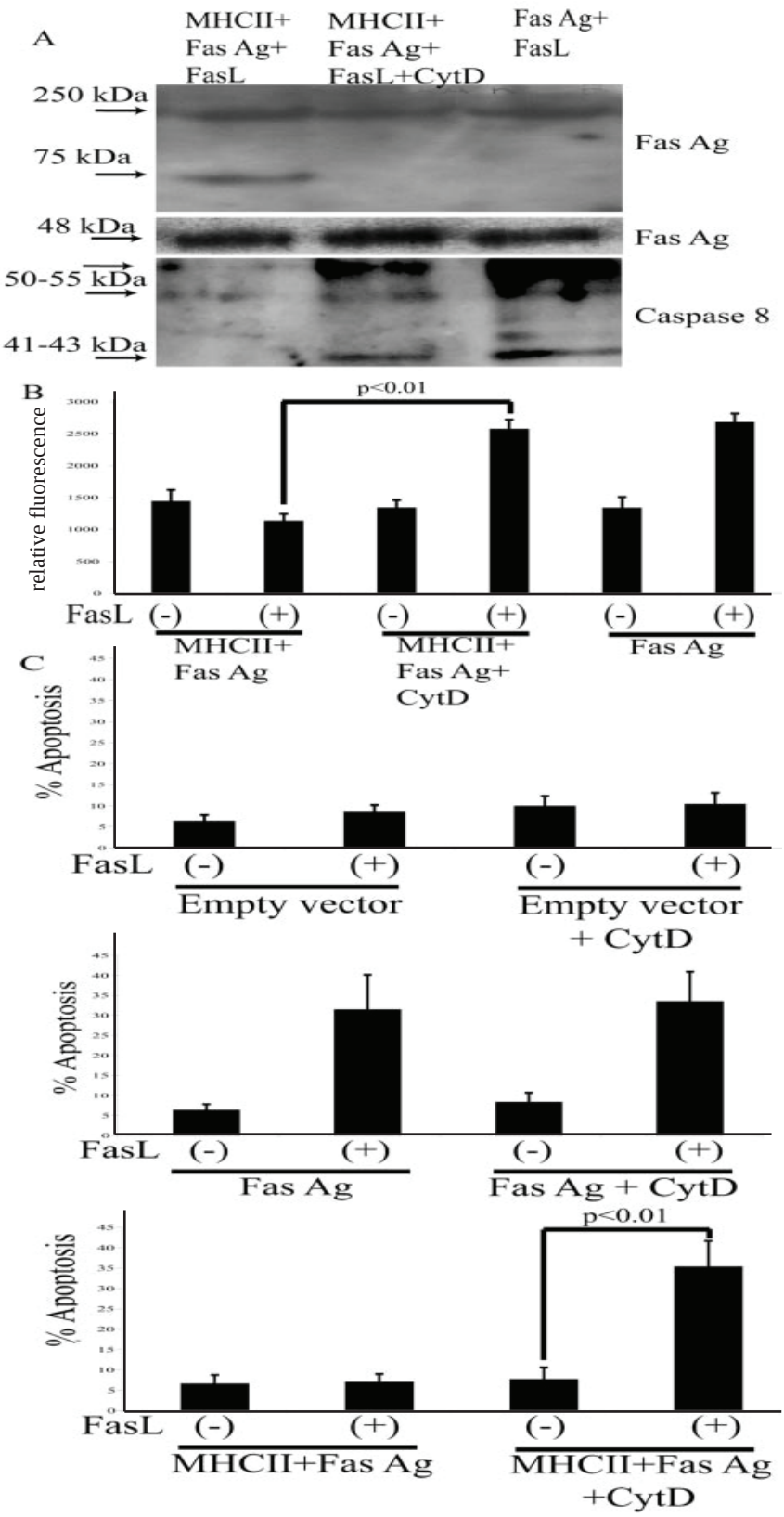


FIGURE 3.8 MHCII INHIBITS FAS AG SIGNALING THROUGH AN ACTIN-DEPENDENT INHIBITION OF RECEPTOR AGGREGATION.

(A) Cells were cultured with FLAG-tagged Fas L at 25 ng/ml for 15 min. DISCs were isolated from MHCII/Fas Ag-expressing cells (lane 1), MHCII/Fas Ag-expressing cells grown in the presence of cytochalasin D at 0.5 μg/ml to inhibit actin polymerization (lane 2), or Fas Ag-expressing cells (lane 3). Proteins were run on a nonreducing gel and blotted with anti-Fas Ag antibody. Monomeric complexes are seen at 75 kDa, and aggregated complexes are seen at 250 kDa. Bands were confirmed to be specific for Fas aggregates by running an aliquot of the sample on a reducing gel and blotting for Fas Ag (middle gel) or caspase 8 (bottom gel). **(B)** Cell lines were grown in the presence or absence of Fas L at 25 ng/ml for 1 h as indicated, and caspase 8 activity was measured and is presented as relative fluorescent units. Bars represent averages from three experiments ±1standard deviations. **(C)** Control cells (empty vector) (top panel), Fas Ag-expressing cells (middle panel), and MHCII/Fas Ag-expressing cells (bottom panel) were grown in the presence or absence of cytochalasin D, with or without Fas L at 25 ng/ml, for 4 h as indicated, and apoptosis was measured by annexin V staining. Each bar represents the average from three experiments ±1 standard deviation.

activation was restored to the same level as seen in cells expressing Fas Ag only (Fig. 3.8B), and the addition of CytoD restored the Fas L-induced apoptotic response (Fig. 3.8C) while not inducing an increase in spontaneous death in the absence of ligand. CytoD had no effect on the basal level or Fas L-mediated apoptosis in the parent RGM-1 cell line or in cells expressing high levels of Fas Ag alone (Fig. 3.8C).

Summary

Dysregulation in the balance between proliferation and apoptosis is felt to underlie the development of cancer; it is not that inhibition of apoptosis per se is carcinogenic, but rather that inhibition of apoptosis in cells which otherwise would have been eliminated increases the likelihood of accumulating harmful genetic defects, leading to a proliferative advantage and the emergence of neoplastic clones (103, 125). In the setting of normal inflammation, that is, inflammation associated with acute injury and wound healing, environmental alteration due to chemokines and cytokines is a self-limited event (125). With chronic inflammation, such as is seen with *Helicobacter* infection, there is constant exposure of the mucosa to cytokines and chemokines, leading to dysregulation of growth (19, 59, 60). Growth programs for repair and proliferation are activated, and apoptotic programs may be bypassed in an attempt to restore mucosal integrity. This sedition of the normal cell death and repair programs results in abnormal DNA replication and proliferation, pushing cells to acquire mutations (125). Because Fas-mediated apoptosis is a central feature of cell loss in *Helicobacter* infection (12, 19, 59, 60, 62, 81, 85, 113, 115, 119, 120, 123, 126-129), alterations in the regulation of this

pathway have great potential for contributing to the malignant process. Here we show that one mechanism of apoptosis escape in the inflamed stomach is through the acquisition of MHCII expression and its subsequent interaction with Fas Ag signaling cascade. Fas Ag signaling is well recognized as a mediator of gastric mucosal apoptosis and plays a central role in *Helicobacter* induced mucosal injury (81, 83, 114, 115, 127, 128). In the face of inflammation, Fas Ag is highly expressed in numerous cell types within the gastric mucosa, including invading inflammatory cells and gastric epithelial cells, with the highest expression seen in chief and parietal cell compartments (114). Certainly, surface Fas Ag appears to be necessary for parietal cell loss and atrophy (113, 114), which are early and progressive events in *Helicobacter* disease (81), culminating in gastric mucosal atrophy. In the setting of atrophy, there is peripheral stem cell failure and an influx of bone marrow-derived stem cells in an apparent reparative effort (123). These marrow-derived cells engraft and differentiate toward an incomplete gastric cell phenotype, forming the basis of metaplastic and dysplastic glands. While most cells expressing high receptor abundance (specifically parietal and chief cells) undergo apoptosis early in infection (59, 114), these metaplastic and dysplastic cell appear to be resistant to apoptotic signaling (86, 87). This phenotype of apoptosis resistance in the setting of high receptor abundance suggests that a mechanism other than receptor number is used to regulate susceptibility to Fas signaling events in these cells and may involve several points of regulation along the Fas signaling cascade. While much is known about Fas signaling in immune cells, much less is known about signaling regulation in other cell types. In general, Fas-mediated cell signaling events are controlled by an abundance of independent mechanisms, including but not limited to factors which alter the

expression level and location of Fas Ag, availability of ligand, assembly and function of the DISC, and regulation of caspase activation and function. Integration of a wide variety of extracellular and intracellular events in the context of cell type specificity confers a high degree of flexibility to Fas L/Fas Ag signaling outcomes. We have previously shown that one level of regulation of the Fas pathway within the gastric mucosa is through modulation of receptor number. High levels of IL-1β, TNF-α, and IFN-γ, found in the infected gastric mucosa, regulate surface Fas Ag expression (85) and are associated with more severe diseases states (59, 60, 62), due in part to increased Fas signaling. Interestingly, this same environment that increases Fas Ag expression (most notably, elevated IFN-γ levels) regulates MHCII expression in gastric mucosal cells, albeit with different levels of protein expressed in different cell types. This differential expression of Fas Ag and MHCII within the gastric mucosa creates the potential for receptor interaction and variable signaling outcomes. MHCII is expressed in metaplastic and dysplastic tissue (unpublished observation) as well as in carcinoma (119, 130, 131), making a scenario of MHCII/Fas Ag receptor interaction plausible. As is true with other aspects of Fas signaling, the interaction between the MHCII complex and the Fas Ag pathway is cell type specific and appears to be unique to each situation studied. For example, in lymphoid cells, MHCII expression paradoxically increases Fas-mediated signaling in B-lymphocytes, where MHCII expression is a cofactor in B-cell-mediated apoptosis through the production of “second messengers” (132) the identities of which are incompletely defined (133). Quite the opposite, MHCII ligation via interaction with T cells confers rapid Fas resistance in B cells (134) and in T cells, where CD4 and MHCII

interactions are required for survival of resting CD4 cells; coexpression offers resistance against Fas-mediated apoptosis (135, 136). Immature dendritic cells, which express moderate levels of Fas surface receptor and low levels of or absent MHCII, are highly sensitive to Fas-mediated apoptosis. As these cells mature and express high levels of MHCII, they become fully resistant to Fas apoptotic signaling (136), while maintaining sensitivity to MHCII-induced apoptosis.

H. pylori induced chronic inflammation may promote mechanisms to protect against Fas induced apoptosis. MHCII is upregulated on the surface of gastric epithelial cells when stimulated with IFN-γ in vitro or on the gastric cells in vivo. Indeed, we found a population of gastric cells that coexpress both, MHCII and Fas Ag. MHCII expression is inducing actin-dependent cytoskeletal changes that impair Fas Ag receptor aggregation and therefore, resistance to apoptosis.

CHAPTER IV. COINFECTION MODULATES INFLAMMATORY RESPONSES AND CLINICAL OUTCOME OF *HELICOBACTER FELIS* AND *TOXOPLASMA GONDII* INFECTIONS

Introduction

Chronic infections are the cause of considerable morbidity and mortality. Unlike acute infections, in which the "one microbe-one disease" concept can be applied, disease caused by chronic infectious organisms more likely represent interactions between the infecting organism(s) and a multitude of host and environmental factors including interaction with other infectious agents. Using a mouse model of infection we examined the interaction between *Helicobacter felis* and *Toxoplasma gondii*: two ubiquitous organisms that cause a spectrum of clinical disease. The host immune response is not able to eliminate *H. pylori*, and infection persists for the life of the host, setting up a situation whereby the gastric mucosa, which is normally devoid of inflammatory cells, becomes chronically inflamed(137). Cytokines present within the gastric mucosa are thought to determine the type and severity of damage. Neither a Th1 nor Th2 response eliminates the bacterium, however the two responses are associated with very different outcomes in terms of gastric mucosal disease.

A Th1 cytokine pattern is associated with parietal cell loss, atrophy, metaplasia, dysplasia, and progression to adenocarcinoma, whereas a Th2 pattern is associated with a relative lack of architectural alterations and cell loss, and there is no progression of disease (64, 138-140). Although host genetics certainly play a role in determining the direction and vigor of the immune response, infections with other organisms that augment or suppress the Th1 response may potentially exacerbate or reduce disease severity, respectively. The "African enigma" (high rates of infection and low gastric cancer rates) has been explained in part by modification of the immune response by

concurrent helminthic infection (65), which affects a “switch” in Th1/Th2 polarity and confers a degree of protection. In contrast to the protective effects of helminthic infection, we reasoned that chronic infections that evoke a predominant Th1 response (such as *T. gondii*) would modulate the immune response to accelerate *Helicobacter* disease.

Toxoplasmosis is caused by the obligate intracellular protozoan parasite *T. gondii*. Infection is common in children and young adults and infection rates range from 10% to over 90% dependent upon many factors including climate, dietary habits, and animal exposure. *T. gondii* induces a strong IFN-γ response that limits the growth of parasites in peripheral tissues, and directs the transformation of parasites from the tachyzoite stage into dormant cysts. Dormant cysts are kept quiescent in chronic latent infection by a continued immune response (141). Impairment of this specific cell mediated response, as seen in immunocompromised patients, leads to a more aggressive acute infection or in the case of latent infection, reactivation of dormant cysts and reemergence of acute disease (142). In the absence of overt immunosuppression, it is not clear what factors if any influence the outcome of acute or chronic *T. gondii* infection. Although population based studies are sparse, there appears to be a positive correlation between *T. gondii* Ab production and gastric cancer (143-146), suggesting that *T. gondii* infection may influence the clinical outcome of *Helicobacter* infection.

This study was initiated to examine the interaction between *T. gondii* and *H. felis* infection using the BALB/c mouse, a strain resistant to disease caused by both organisms. Coinfection with *H. felis* significantly blunted the IFN-γ response to *T. gondii*, allowing

considerable tachyzoite replication, tissue damage, and increased mortality. Additionally, long-term coinfection leads to a prominent *H. felis*-specific Th1 response, blunting of the *H. felis* specific Th2 response and significant gastric mucosal damage. Collectively, these findings point to a dynamic interaction between immune responses to seemingly unrelated organisms with significant impact on disease outcomes.

Results

1. *BALB/c mice infected with H. felis had higher mortality when challenged with T. gondii.*

The BALB/c strain of mice is easily infected with both *T. gondii* and *H. felis*, but it is considered a resistant host, as it does not develop clinical disease with either organism (147, 148). Control mice and mice infected with *H. felis* alone did not differ in weight, activity, or general well being at any time point of the experiment and there were no mortalities among mice within these groups. Only one mouse infected with *T. gondii* alone (n = 20) died on day 11; the remainder did not develop distress or evident disease. In sharp contrast, mice coinfected with *H. felis* and *T. gondii* became acutely ill as early as day 3 after *T. gondii* infection. Mice with established *H. felis* infection (n = 20) 20 *wk* before *T. gondii* infection developed signs of dehydration, bloody stool, huddling, and decreased activity with 50% of the mice dying between day 7 and day 15. Of those that survived, recovery was evident by day 17, after which all remained well and without distress for the duration of the experiment. To assess the effects of acute *H. felis*

infection, mice were infected with *T. gondii* first, and on days 5, 7, and 9 (the time of peak IFN-γ response to *T. gondii*) were infected with *H. felis*. Mortality in this group was less than in the chronically infected group, but substantially higher than *T. gondii* alone, with 25% mortality occurring between day 7 and day 11 (Fig. 4.1A). These mice appeared clinically similar to the *T. gondii* and chronically infected *H. felis* mice, although less severely affected, with dehydration, decreased activity, and huddling. There was complete recovery of survivors by day 17.

2. Concomitant H. felis infection causes a blunted IFN-γ response to T. gondii and higher parasite loads.

Host susceptibility to *T. gondii* infection is determined by the vigor of the immune response. Too strong an IFN-γ response induces a septic shock-like picture whereas an inadequate response allows tachyzoite replication and widespread tissue damage. BALB/c mice normally respond with modest IFN-γ induction; adequate to control tachyzoite replication yet low enough to avoid systemic complications. Control mice and those infected with *H. felis* alone did not have detectable peripheral IFN-γ levels (Fig. 4.1B). With *T. gondii* infection alone, peripheral blood IFN-γ levels peaked between days 3 and 11 and steadily declined thereafter to below the level of detection by 4 wk. Mice that were coinfected with *H. felis* and *T. gondii* had significantly lower IFN-γ levels when compared with mice infected with *T. gondii* alone and IFN-γ was most pronounced in mice that had a chronic *H. felis* infection at the time of *T. gondii* challenge. In the

FIGURE 4.1 CONCURRENT *HELICOBACTER* INFECTION BLUNTS THE IMMUNE RESPONSE TO *T. GONDII* AND INCREASES *T. GONDII* RELATED MORTALITY.

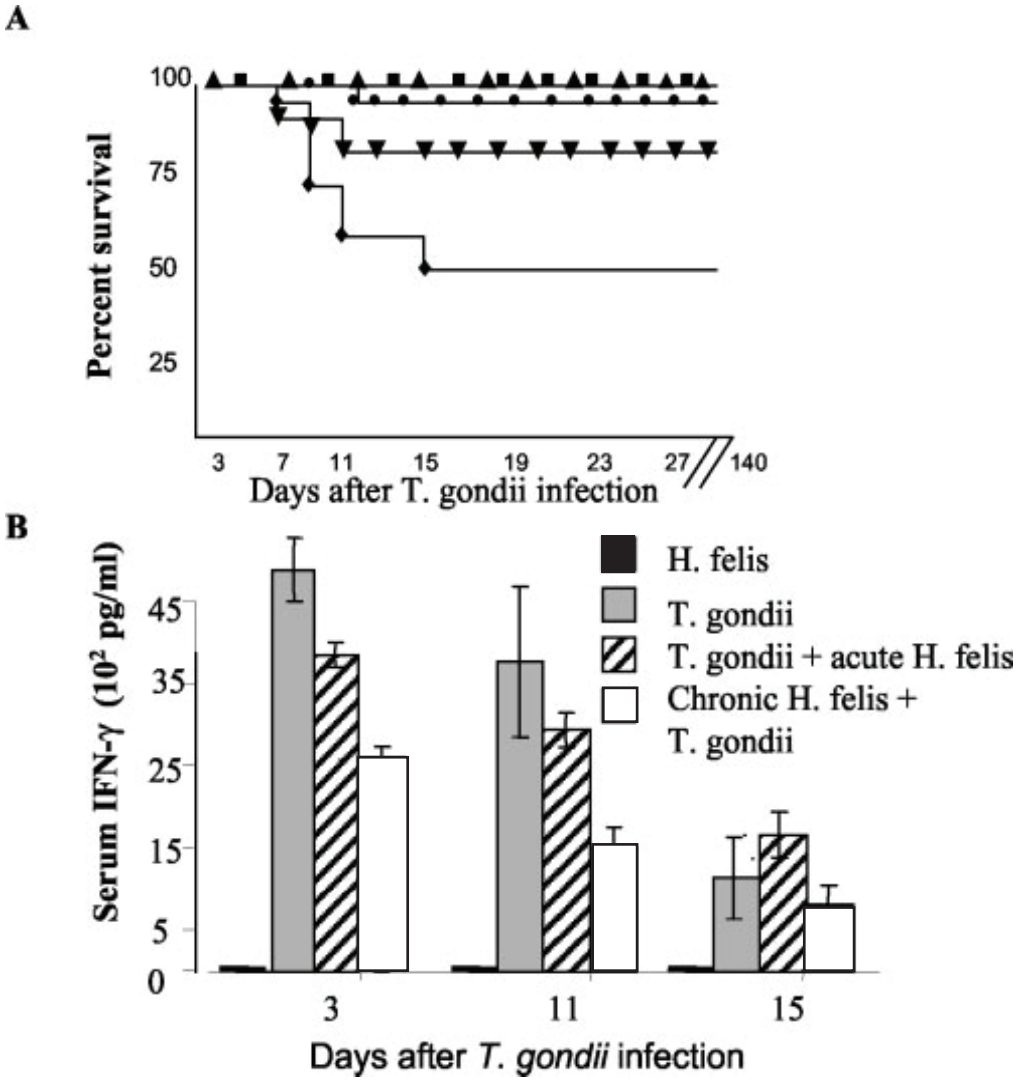


FIGURE 4.1 CONCURRENT *HELICOBACTER* INFECTION BLUNTS THE IMMUNE RESPONSE TO *T. GONDII* INFECTION AND INCREASES *T. GONDII* RELATED MORTALITY.

(A) Kaplan-Meier survival curves after *T. gondii* challenge in mice infected with *H. felis* or sham infected. Twenty mice in each group were followed for 20 wk after *T. gondii* infection. The experiment was repeated a second time (with 10 mice each) with similar results. Control (▲), *H. felis* (■), *T. gondii* (●), *T. gondii* + acute *H. felis* (▼) infection, *T. gondii* +chronic *H. felis* (◆) infection. **(B)** In a second cohort of mice, serum IFN-γ levels were determined by ELISA at 3, 11, and 15 days post *T. gondii* infection (n=5 for each group). Data from control mice and *H. felis*-infected mice were below the level of detection and data from these groups combined. After day 15, serum IFN-γ was below the level of detection in all groups. Results are reported as the mean ±1 SD. The experiment was repeated a second time with similar results.

surviving mice, IFN-γ levels steadily declined becoming undetectable after 4 wk and remained undetectable for the duration of the study in all groups. We next evaluated whether lower IFN-γ levels were associated with higher parasite replication, by examining the spleen parasite load in mice days 3 or 11 after infection. Control mice and mice infected with *H. felis* alone had undetectable *T. gondii B1* gene expression, and no visible tachyzoites on H&E stained sections (Fig. 4.2A–C). Mice infected with *T. gondii* alone had moderate *B1* gene expression in the spleen and occasional tachyzoites visible on histological section (Fig. 4.2, A, B, and D). In sharp contrast, mice that were chronically infected with *H. felis* before *T. gondii* challenge had a 3-fold increase in *T. gondii B1* gene expression (Fig. 4.2A) and grossly visible tachyzoites in splenic tissue (Fig. 4.2E) accompanied by areas of infarction and necrosis.

The small bowel of control mice and of mice infected with *H. felis* alone was unremarkable. Examination of the distal small bowel of *T. gondii* infected mice was grossly similar to control mice, with the exception of occasional prominent elevations on the serosal surface consistent with enlarged lymphoid aggregates. Gross examination of the luminal surface was within normal limits. Microscopically there was mild to moderate collections of acute inflammatory cells within the lamina propria of the ileum, preservation of architecture, and no visible tachyzoites (Fig. 4.2F). In sharp contrast, the small bowel of dual-infected mice had areas of gross necrosis, hemorrhage, and edema. Necropsy on those that were moribund or had died showed evidence of bowel perforation with peritonitis evidenced by free fluid in the peritoneal cavity, adherence of bowel loops to each other, and free fecal matter in the peritoneal cavity. Microscopically, there was

FIGURE 4.2 MICE WITH CONCURRENT *H. FELIS* INFECTION HAVE HIGHER PARASITE LOADS AND TISSUE DAMAGE WITH *T. GONDII* INFECTION.

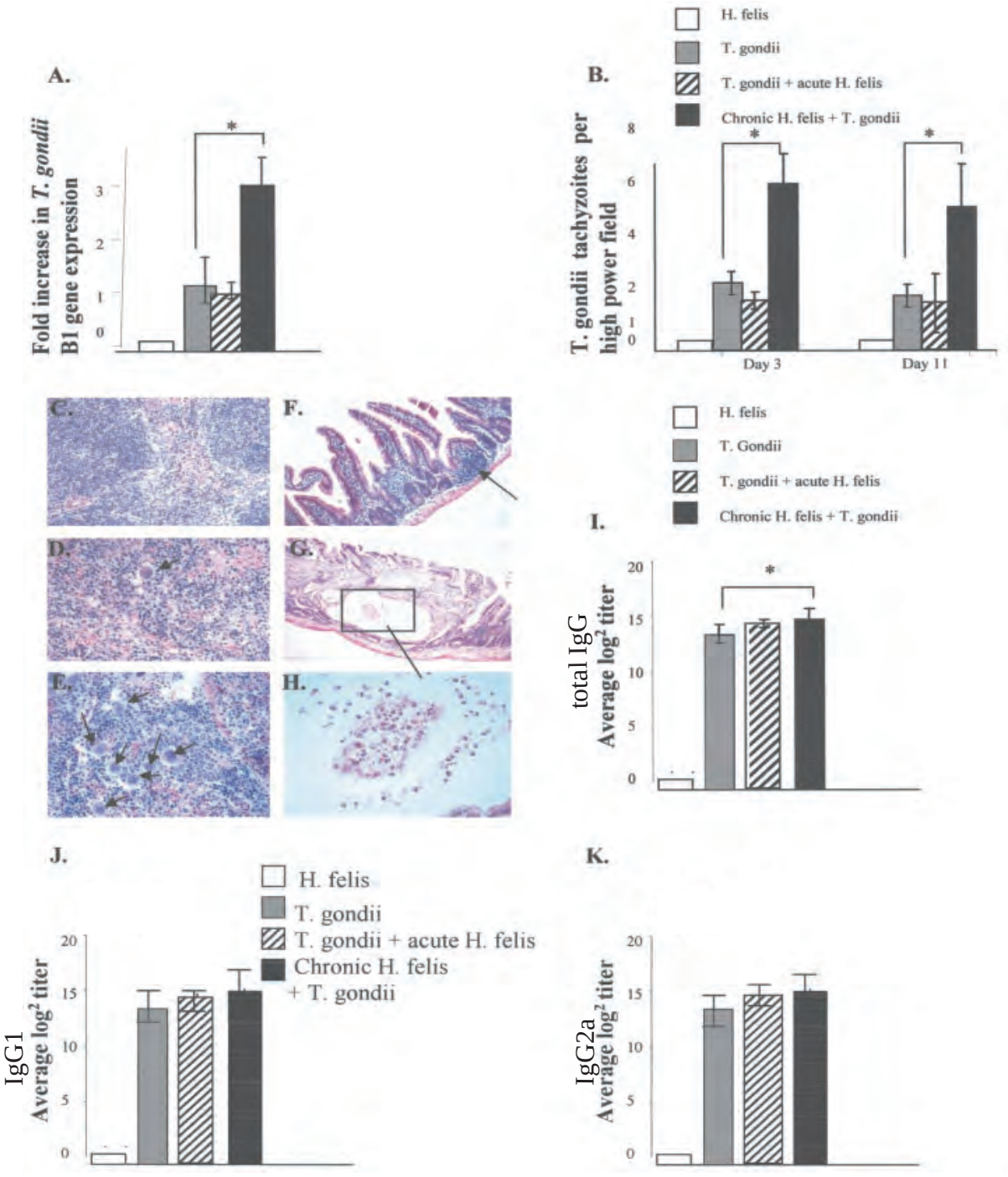


FIGURE 4.3 *T. GONDII* INFECTION ALTERS THE *H. FELIS*-SPECIFIC ANTIBODY RESPONSE.

Serum levels of *H. felis*-specific IgG1 and IgG2a were measured by ELISA at 7 wk **(A)**, 20 or 40 wk **(B)** post-*H. felis* infection (n =10 for each group). Error bars represent 1 SEM, * p =0.0012, ** p =0.031, *** p =0.04.

marked inflammation of the lamina propria, villus blunting, and architectural distortion. Visible tachyzoites were seen proliferating within cystic lesions (Fig. 4.2, G and H). At 20 wk of *T. gondii* infection, the total *T. gondii*-specific serum IgG Ab response was significantly higher in those mice that had an established *H. felis* immune response before *T. gondii* challenge compared with those mice infected with *T. gondii* alone (Fig. 4.2I), however there were no differences in the ratio of IgG1 to IgG2a when subgroup analysis was performed (Fig. 4.2, J and K).

3. T. gondii infection induces an H. felis–specific IgG2a/IgG1 isotype switch.

H. felis infection altered the acute response to *T. gondii* by blunting the IFN-γ response and allowing uncontrolled parasite replication. We next addressed the impact of *T. gondii* infection on the *H. felis*-specific response, with the prediction that high levels of IFN-γ generated by *T. gondii* infection would induce a prominent and sustained bias toward a local gastric *H felis*-induced Th1 cytokine profile. We measured *H. felis*-specific IgG2a and IgG1 Abs at an early time point of infection (7 wk) and times representing chronic infection (after 20 wk coinfection) in mice that were infected with *H. felis* and *T. gondii* at the same time and in mice who had established *H. felis* infection at the time of *T. gondii* exposure. There was a significant increase in *H. felis*-specific IgG2a levels ($p<0.0012$), and a concomitant decrease in IgG1 Ab when *T. gondii* infection was present compared with the findings in *H. felis* infection alone. Ab were not detected in control mice, nor in mice infected with *T. gondii* alone (Fig. 4.3A). This response was

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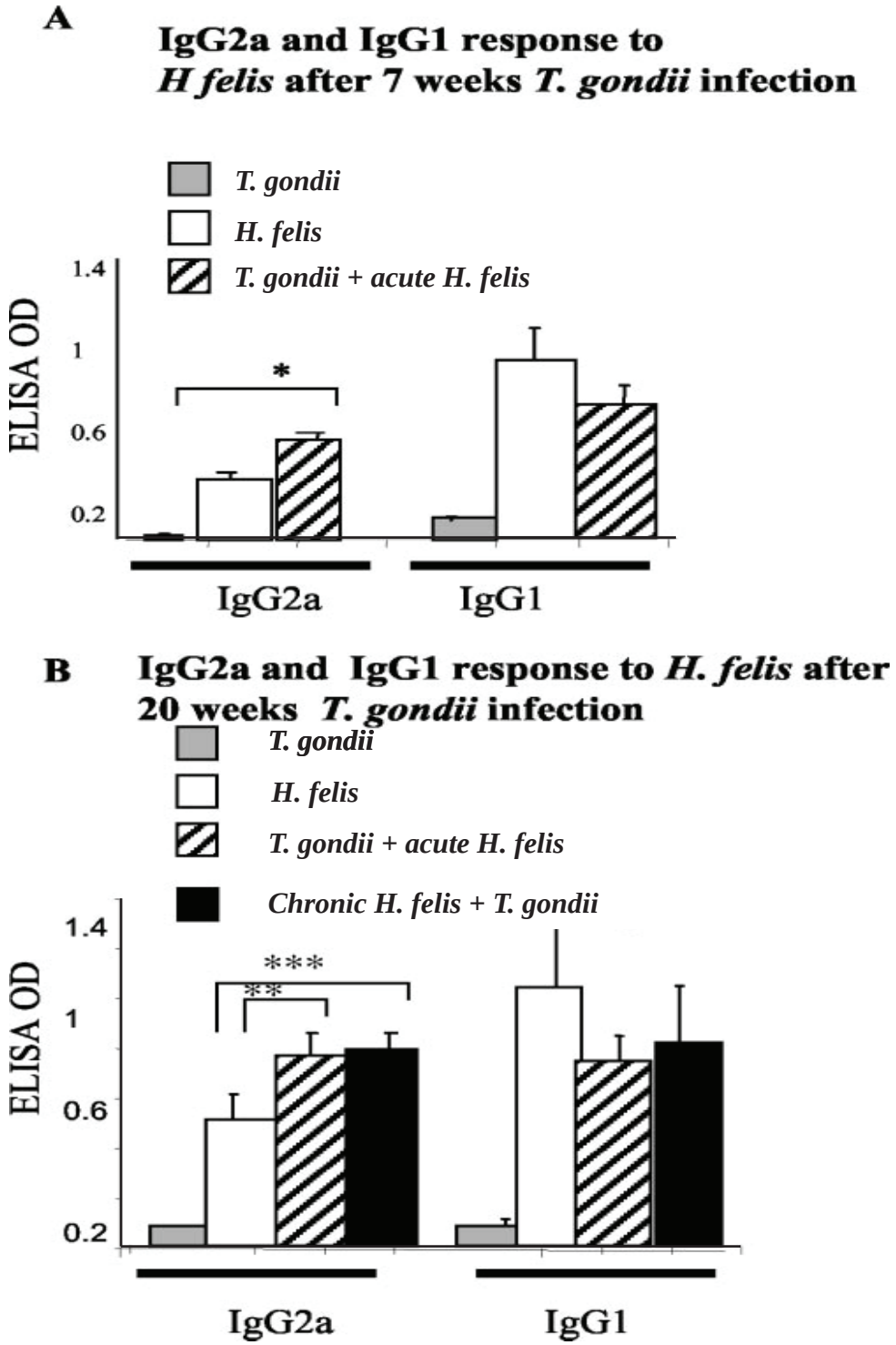

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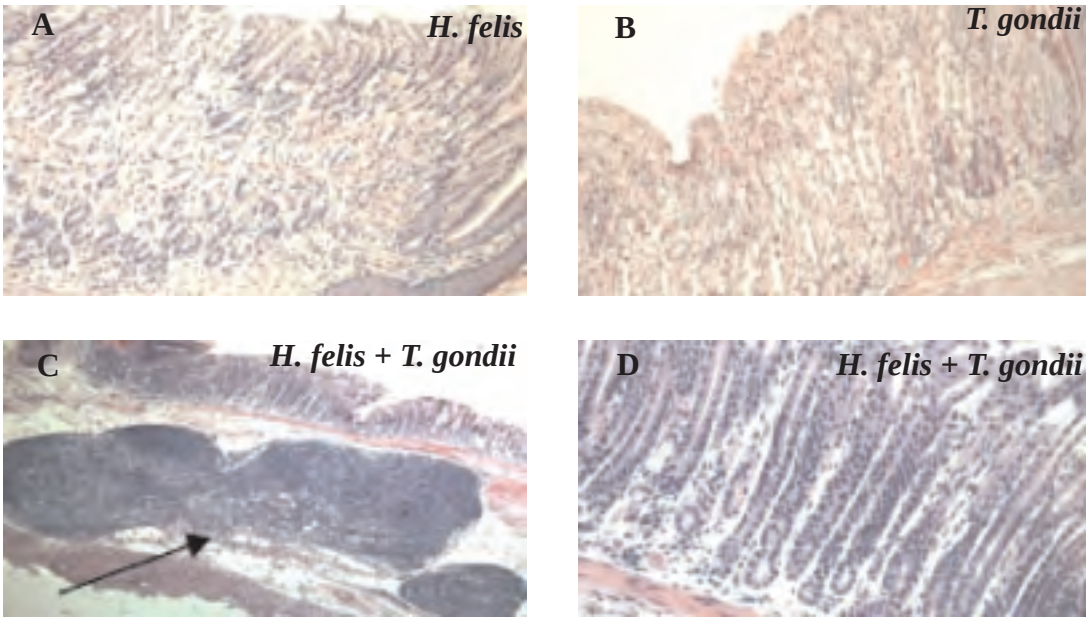
Serum levels of *H. felis*-specific IgG1 and IgG2a were measured by ELISA at 7 wk **(A)**, 20 or 40 wk **(B)** post-*H. felis* infection (n =10 for each group). Error bars represent 1 SEM, * p =0.0012, ** p =0.031, *** p =0.04.

maintained for the duration of the experiment, with significant increases in anti-*H. felis* IgG2a levels maintained in the concurrently infected group ($p<0.031$). Significantly, mice that had an established *H. felis* infection before *T. gondii* challenge, substantially increased production of IgG2a Ab ($p<0.04$), while maintaining the trend toward decreased IgG1 Ab production (Fig. 4.3B).

4. T. gondii infection increases H. felis gastritis and mucosal damage.

Mice infected with *H. felis* for 20 wk had mild corpus gastritis confined to the lesser curvature at the squamocolumnar junction, but were otherwise normal. The inflammation was characterized by a chronic inflammatory cell infiltrate composed mostly of lymphocytes appearing singly, or occasionally in small aggregates at the base of glands in the submucosa. There were no architectural alterations in the gastric mucosa, with preservation of gland structure, normal mucosal thickness and normal parietal cell mass (Fig. 4.4A). Mice infected with *T. gondii* alone for 20 wk had no alterations in the gastric mucosa. Specifically, there was little inflammation, preservation of mucosal thickness and no change in parietal or chief cell number (Fig. 4.4B). In contrast, mice infected with *H. felis* and *T. gondii* together had severe gastritis extending throughout the mucosa most severe in the corpus, but present in the antrum as well. Inflammation was characterized by a chronic inflammatory cell infiltrate composed mostly of lymphocytes extending throughout the thickness of the mucosa and forming discrete nodules in both the mucosal and submucosal areas. Some lymphoid aggregates were massive, distorting the overlying mucosa (Fig. 4.4C) and were grossly visible at the time of necropsy.

FIGURE 4.4 *T. GONDII* INFECTION ALTERS THE GASTRIC MUCOSAL RESPONSE TO *HELICOBACTER* INFECTION.



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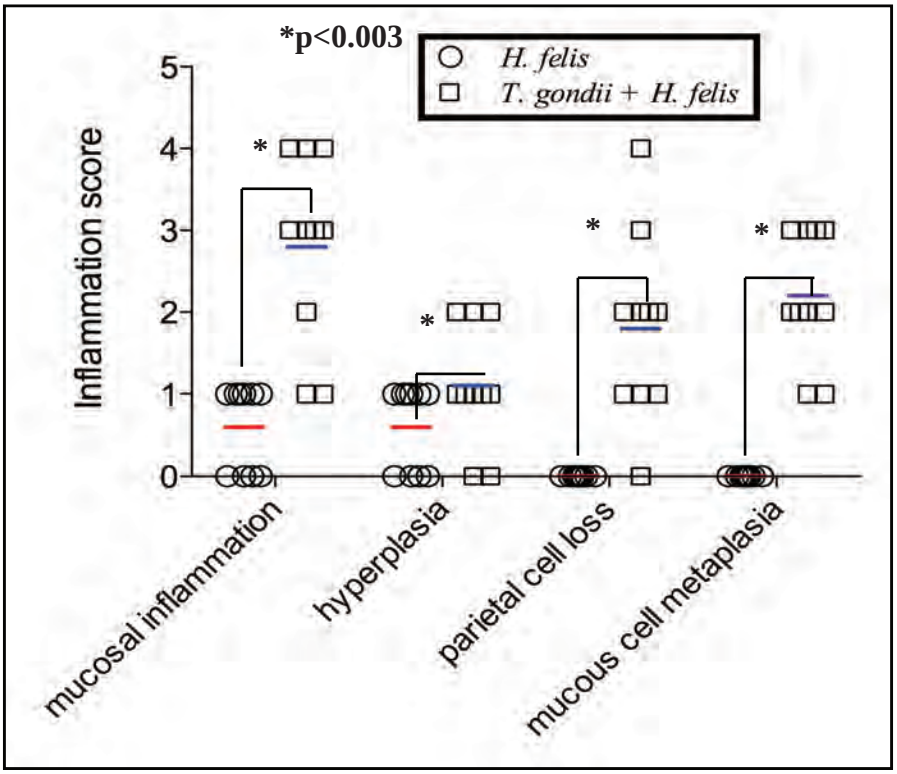


FIGURE 4.4 *T. GONDII* INFECTION ALTERS THE GASTRIC MUCOSAL RESPONSE TO *HELICOBACTER* INFECTION.

All sections are representative of findings at 20 wk. **(A)** *H. felis* infection in the BALB/c (group 2) mouse does not cause mucosal damage. Architecture is preserved, with occasional lymphocytes noted in the deep mucosa. **(B)** *T. gondii* infection alone does not cause gastric mucosal damage. The gastric mucosa of *T. gondii*-infected mice did not differ from control mice. **(C)** *T. gondii* infection concurrent with *H. felis* infection results in massive lymphocytic infiltrates in both the submucosa (arrow) and within the mucosa. Architectural distortion is prominent with loss of parietal and chief cells, antralization of glands and mucous cell metaplasia. Antralization and parietal cell loss is shown at higher power in **(D)**. **(E)** Lesions were scored as outlined in *Materials and Methods* for 10 mice in each group. Number of symbols equals the number of mice achieving that score. Scores were compared using a two-tailed Mann-Whitney analysis of nonparametric data and *p* values are shown at *top* of each column. Magnification, X20 **(A and B)**, X10 **(C)**, and X40 **(D)**. *$p<0.003$ – statistically significant.

Infection with both *H. felis* and *T. gondii* was associated with considerable diffuse glandular epithelial atrophy, with extensive replacement of parietal cells and moderate antralization of the glands within the corpus (Fig. 4.4D). Lesion scores are presented in Fig. 4.4E.

5. Dual infection alters level of H. felis colonization.

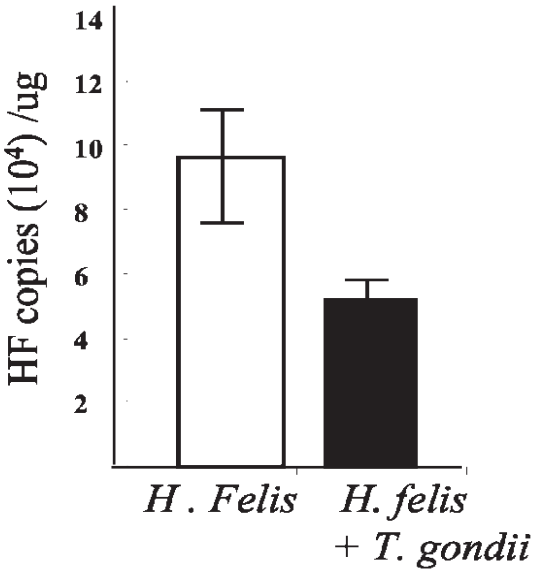
H. felis infection was confirmed by serum Ab determination, histological detection of organism, and by DNA quantification in all mice experimentally infected. Organisms were not detected in those mice not specifically infected. *H. felis* colonization was highest in those mice infected with *H. felis* alone, compared with those mice coinfected with *T. gondii* (Fig. 4.5A). These findings are consistent with published reports of higher bacterial loads associated with Th2 responses and lower bacterial loads associated with a Th1 immune response (4).

6. Gastric mucosal cytokine levels are altered in mice coinfected with T gondii compared with those infected with H. felis alone.

In both human and mouse infection studies, the local cytokine environment predicts disease outcomes (201), with high IL-1β and low IL-10 predictive of severe disease in humans, and the combination of high IFN-γ levels and low IL-10 permissive for severe mucosal damage (61, 64, 149) in various mouse models. We therefore

FIGURE 4.5 CONCURRENT *T. GONDII* INFECTION SHIFTS THE LOCAL GASTRIC MUCOSAL CYTOKINE RESPONSE TO *H. FELIS* FROM A TH2 PROFILE TO A TH1 PROFILE.

A



B

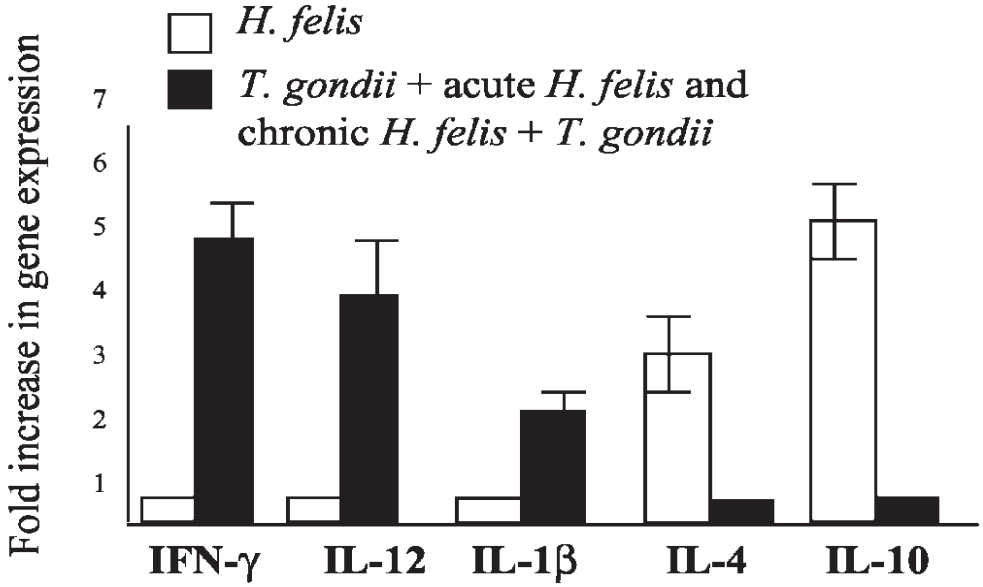


FIGURE 4.5 CONCURRENT *T. GONDII* INFECTION SHIFTS THE LOCAL GASTRIC MUCOSAL CYTOKINE RESPONSE TO *H. FELIS* FROM A TH2 PROFILE TO A TH1 PROFILE.

(A) Quantitative PCR for *H. felis* infection reported as number of bacteria (as a function of gene copy number) per microgram of total gastric tissue. Error bars represent 1 SD (n=10 for each group). **(B)** Quantitative PCR of IFN-γ, IL-12, IL-1β, IL-4, and IL-10 in the gastric fundic mucosa of *H. felis*-infected (7 and 20 wk infection time points combined) and *H. felis*/*T. gondii*-infected (7 and 20 wk of *H. felis* infection combined) mice. Results in the dual-infected mice (*Helicobacter*/*T. gondii* each 7 wk, *Helicobacter*/*T. gondii* each 20 wk, and *Helicobacter* 20 wk/*T. gondii* 7 wk) were indistinguishable from each other; therefore data from acute and chronic *Helicobacter* infection were combined. Each sample was run in triplicate, values differed by <1%, and an average for each mouse was used in calculation. Results are mean values +1SD (n=10). Experiment was performed two times with similar results.

evaluated the gastric mucosal cytokine production using real-time PCR for IFN-γ, IL-12, IL-1β, IL-4, and IL-10. Proinflammatory cytokine expression in the gastric mucosa of BALB/c mice infected with *H. felis* alone did not differ from levels found in control mice (20 wk time point, *n* =10 for all groups). In sharp contrast, BALB/c mice infected with *H. felis* alone had markedly elevated IL-10 and IL-4 levels (Fig. 4.5B). These findings are consistent with the BALB/c phenotype as a *Helicobacter* resistant host. In sharp contrast, mice coinfected with *T. gondii* and *H. felis* had significant elevations in IFN-γ (5-fold), IL-12 (4-fold), and IL-1β (2-fold) compared with *H. felis* alone regardless of whether the mouse was infected with *H. felis* before, or after the time of *T. gondii* infection. IL-4 expression (3-fold decrease) and IL-10 expression (5-fold decrease) were blunted relative to the robust response seen in the *H. felis*-infected mice (Fig. 4.5B).

Summary

Disease manifestations of chronic infections depend on a multitude of interacting factors specific to the host, the infecting organism and the environment. In most cases, a level of balance is reached whereby a continued immune response, while not able to fully eliminate an organism, keeps infection in check. The organism may then go undetected until reactivation of infection with suppression of this specific immune response (as occurs with Toxoplasmosis) or in some cases a chronic smoldering infection persists (such as is seen with *H. pylori*), which may show periodic flares of activity, or continually progress to debilitating disease. In the case of *Helicobacter* infection, clinical

outcomes differ widely, with some patients infected but without obvious disease manifestations, whereas others develop severe manifestations such as gastroduodenal ulceration and gastric adenocarcinoma. The range in disease manifestations has not been fully explained by differences in bacterial factors or host genetics, but may be explained by interactions with other infectious agents modulating the organism-specific immune response. We infected mice with both *T. gondii* and *H. felis* and show the interplay between immune responses was not unilateral, but rather the immune response and clinical outcome to both organisms was affected. This immune modulation was durable resulting in both short-term morbidity and mortality due to *T. gondii* as well as severe chronic mucosal disease secondary to *H. felis*.

The CD4 T cells within the adaptive immune response can be divided into two functional groups, Th1 and Th2 cells, based on the types of cytokines they produce and the immune responses they coordinate. Th1 cells produce IFN-γ, IL-2, and IL-12 and coordinate the cell-mediated immune response. Th2 cells predominantly produce IL-4, IL-5, and IL-10, and induce B cell activation. The direction of maturation of a naive T cell depends upon both the stimulus and the environment in which it is stimulated. High levels of IFN-γ at the time of T cell activation will promote a Th1 phenotype, and high levels of IL-4 will favor a Th2 phenotype. Although individual T cells can be thought of as Th1 or Th2 cells, assigning such a designation to the composite immune response is more complicated, and it should be viewed more correctly as falling on a continuum between the two responses, with the degree of polarization dependent on a variety of factors including properties of the infecting organism, host genetics, and environmental

conditions. As such, there is the potential for manipulation by factors that nudge the response toward one or the other pole, with the possible effect of altering the disease outcome. Looking at mouse models of infection, the C57BL/6 mouse responds to *H. felis* with a Th1 immune response associated with considerable mucosal inflammation, atrophy of the fundic mucosa with loss of parietal and chief cells (59, 61, 62, 65, 81, 93), and the appearance of metaplastic changes progressing to dysplasia. The immune response and premalignant changes closely parallel the events preceding development of gastric adenocarcinoma in humans, making the mouse a powerful model in which to study *helicobacter* infection.

In contrast to this susceptible mouse model, the BALB/c strain of mice develops a Th2 immune response to *H. felis* infection, minimal gastritis, and virtually no histological alterations despite dense colonization with bacteria (139). These two mouse strains represent the extreme poles of immune response to infection, providing models within which to determine the impact of modifiers on the immune response to *Helicobacter* and their subsequent effects on disease initiation and progression. To support the notion of coinfectious agents altering the outcome of disease, it has been shown that concurrent helminthic infection skews the *Helicobacter* specific immune response of the C57BL/6 mouse toward a Th2 profile and protects against short-term disease (65). It is therefore conceivable that infectious or environmental influences may augment a Th1 response in a similar fashion, thereby promoting disease in an otherwise resistant host. The vast majority of individuals do not develop clinical disease with *Helicobacter* infection, suggesting a situation in which a few may be “pushed” toward disease rather than the majority protected from disease. Therefore, perhaps more compelling than protection, are

situations that may increase disease severity. *T. gondii* infection is very common, with evidence of Ab production in every population studied, albeit at varying levels. Infection with *T. gondii* universally induces a strong Th1 response. We reasoned that if high levels of IFN-γ were present at the time the host was mounting an immune response to *H. felis*, the *H. felis*-specific response would shift toward a Th1 cytokine profile.

We further reasoned that a Th1 cytokine profile in an otherwise "resistant" host would be associated with mucosal damage. Indeed, *T. gondii* infection at the time of *Helicobacter* infection effectively converts a resistant host to a susceptible host. Cytokines within the gastric mucosa show a dramatically altered pattern, with gastric IFN-γ levels comparable to those seen in the infected C57BL/6 mouse and a marked diminution in IL-10 levels. Both IFN-γ (64, 149) and IL-10 (61, 68) are pivotal in the gastric mucosal disease process secondary to *Helicobacter* and are felt to be essential factors in disease susceptibility in the mouse model as well as in human disease. Mice that were infected with both *T. gondii* and *H. felis* had a substantial increase in mucosal inflammation with mucosal hyperplasia, parietal and chief cell loss and mucous cell metaplasia, very similar to what is seen in the susceptible C57BL/6 mouse and in humans. It is interesting to note that although the mucosal changes were similar to those found in the infected C57BL/6, they were not identical and took longer to develop, suggesting differences between strains in addition to the immune response. For example, the marked antralization seen in the BALB/c (Fig. 4*D*) is not typically seen in the C57BL/6 model, but it is seen in human *H. pylori* infection (150). In humans, antralization is associated with increased proliferation; reduction in Bax expression and

Bcl-2 overexpression implying that antralization may be an important histological marker for cancer risk (151, 152). This shift from a Th2- to a Th1-specific *H. felis* response is not confined to the gastric mucosal compartment, but is systemic. IgG subclass analysis is consistent with a sustained shift toward a Th1 response in all dual-infected groups. Perhaps most important is that mice with an established polarized *H. felis* response were equally susceptible to immune modulation by *T. gondii*, and the switch in Th1/Th2 polarization was at least as great, if not greater than that seen with acute *H. felis* infection, suggesting there is an enormous window whereby factors may influence the outcome of chronic infections. *T. gondii* is a chronic infection and likely exerts continual pressure toward the *H. felis* Th1 response, which allows the response to be sustained. This raises the question of how other organisms may interact with the immune response to *Helicobacter*. The natural history of symptomatic *Helicobacter* infection in humans is one of active ulcers interspersed with various periods of remission. The trigger for this waxing and waning of disease is not known.

Although we have not evaluated the effects of short-term infectious agents on *Helicobacter* immune response, it is tempting to speculate that acute infectious agents may exasperate *H. pylori* disease by nonsustained shifts in the immune response leading to disease “flares” that would theoretically abate as the acute infectious agent causing them was cleared. Alternatively, more sustained shifts in immune response may lead to more sustained changes such as metaplasia, dysplasia, and cancer. Without eradication therapy, *Helicobacter* infection exists for the life of the host, allowing the *Helicobacter*-specific chronic inflammatory response to influence other infectious disease processes.

Helicobacter infection significantly blunted the IFN-γ response to *T. gondii* and increased morbidity and mortality from *T. gondii* acutely. Although the effects of *Helicobacter* infection on the immune response to other organisms was not tested in this study, our findings suggest that the chronic immune response to *Helicobacter* may impact the host response to other infectious agents, implying an impact on both acute and chronic disease processes during the lifetime of the infected host.

CHAPTER V. T-BET KNOCKOUT PREVENTS *HELICOBACTER FELIS*-INDUCED GASTRIC CANCER

Introduction

The link between chronic inflammation and cancer has been long recognized. Paradoxically, not all patients with chronic inflammation will develop cancer. Therefore, identifying immune response differences that predispose to cancer is paramount in identifying at-risk populations, and these differences may serve as therapeutic targets. *Helicobacter* infection causes chronic gastritis in all infected patients, although not all patients will develop clinical disease. Many infected patients will progress to gastric atrophy and mucous cell and intestinal metaplasia, while a smaller number of patients will develop dysplasia and <1% will develop gastric cancer. The incidence of gastric cancer ranges widely between countries (1, 153-155). Research into the mechanism by which *H. pylori* causes gastric disease has focused on three main areas: bacterial, environmental, and host genetic factors. The main body of research suggests that more virulent strains of bacteria and environmental factors that are associated with gastric cancer function via modulation or augmentation of inflammation within the gastric mucosa.

Host genetic factors in turn dictate the intensity and the composition of the inflammatory response such that infected individuals with cytokine polymorphisms favoring a stronger proinflammatory response with elevated levels of TNF-α, IL1-β, and low IL-10 are more likely to develop more severe disease (67, 68). Studies using mouse models have been instrumental in defining the role of inflammation in *Helicobacter* disease progression and have clarified significantly the role of the adaptive immune

response to mucosal damage. Mouse models allow controlled infection studies in genetically identical mice under restricted, regulated conditions. Different mouse strains have distinct immune responses to the bacterium (59, 61) and are very useful for studying various clinical manifestations of *Helicobacter* infection. *H. felis* infection in a genetically susceptible mouse strain, such as C57BL/6J, results in an acute inflammatory infiltrate into the gastric mucosa, which progresses to a chronic inflammatory response over several weeks (93, 102). During this time, parietal and chief cells are lost at least in part through Fas Ag/Fas L signaling (85). Once parietal cells are lost, the normal gastric gland architecture is replaced by a metaplastic mucous cell lineage followed by atrophy and intestinal metaplasia. By 15 months of *H. felis* infection, gastric adenocarcinoma develops in most mice (102). This pattern of mucosal alterations closely resembles the pattern and evolution of human gastric carcinoma (156).

We have shown how *H. felis* infection in a mouse strain that is resistant to *Helicobacter*-induced gastric adenocarcinoma, such as the BALB/c, produces a very different mucosal immune response (61, 157) Mice become colonized by bacteria easily and often support higher bacterial loads than the C57BL/6J mice. Inflammation is initially submucosal with sparse intramucosal infiltrates, as is seen with infection in the C57BL/6J mouse; however, as *H. felis* infection progresses, lymphoid nodules are prominent and few mice may develop MALT (Mucosa-Associated Lymphoid Tissue) lymphoma. Strikingly, parietal and chief cells are largely preserved and atrophy does not develop. While the *H. felis*-infected BALB/c is a useful model of MALT lymphoma, it does not develop gastric adenocarcinoma. The major factor linked to the different outcomes of infection between a susceptible and resistant host is the immune response to

H. felis. Th1 cytokines are associated with gastric adenocarcinoma, and a Th2 response is associated with relative resistance to gastric adenocarcinoma (59, 61). C57BL/6 mice tend to Th1 responses, while BALB/c mice usually polarize to Th2 responses. However, in addition to opposing immune response polarity, these two mouse strains are genetically different, and it is not clear how other genetic differences contribute to disease manifestations.

Data from our laboratory as well as from those of others strongly supports that a vigorous Th1 response is required for disease to occur. IFN-γ infusions via osmotic pump into C57BL/6 mice for up to 4 wk is sufficient to induce inflammation with parietal cell loss and mucous cell and intestinal metaplasia, even in the absence of *Helicobacter* infection (64). With *Helicobacter* present, mucosal damage is greatly accelerated (64). Conversely, dampening a Th1 response greatly attenuates disease in the C57BL/6 mouse; however, it does not completely prevent mucosal damage (65). It is tempting to conclude that blocking the Th1 response in the C57BL/6 model is responsible for disease protection. However, the model used employed a helminthic infection to switch the immune response to *Helicobacter* infection, and direct effects of the helminth or the immune response to the helminth above and beyond the Th1/Th2 switch were not addressed. We have done similar immune manipulations in the BALB/c mice and have shown that overriding the usual Th2 response and forcing a Th1 cytokine pattern can alter disease progression, resulting in mucosal damage reminiscent of (but not identical to) damage in the C57BL/6 model (157) and **CHAPTER IV**. Taken together, these data suggest that manipulations within the C57BL/6 and the BALB/c model greatly alter

inherent disease susceptibility, with a Th1 response necessary for disease progression.

T-bet is a member of the T-box family of transcription factors, and it appears to regulate the commitment of Th cells to the Th1 lineage (158). It does so in part by transactivation of IFN-γ. Additionally, IFN-γ production is markedly impaired in NK cells from these mice. Strikingly, however, T-bet is not involved in controlling IFN-γ production in cytotoxic $CD8^+$ T cells. The T-bet knockout (KO) mouse offers a model where Th1 responses can be directly assessed with minimal alterations of other immune functions, and without the effects of global cytokine deficiency seen in other transgenic models.

Therefore, to definitively address the role of Th1 immune response in *Helicobacter*-induced disease, T-bet KO mice in the C57BL/6 background or their wild-type littermates were infected with *H. felis* and followed for up to 15 mo for disease progression. Both groups maintained *H. felis* infection throughout the study. Analysis of mucosal cytokine patterns and *H. felis*-specific IgG subclass analysis confirmed a blunted Th1 response in the T-bet KO mice. Wild type mice progressed through tissue alterations of metaplasia, atrophy, and dysplasia to carcinoma, while the T-bet KO mice maintained parietal and chief cell populations and mucosal integrity and failed to develop adenocarcinoma. Our data clearly establish a link between IL1-β and TNF-α in the pathogenesis of *Helicobacter*-induced gastric adenocarcinoma. Parallel cytokine patterns in susceptible human populations suggest that differences in T-bet regulation may underlie susceptibility to gastric cancer in human populations.

Results

1. Loss of T-bet does not alter the gastric mucosa in uninfected adult mice.

We examined both male and female mice, five of each gender for each time point. Stomachs of uninfected (sham infected) mice were examined at 4 or 15 months after initiating the experiment (6–17 months of age). Stomachs of uninfected WT (wild type) and T-bet KO mice were grossly indistinguishable and were of similar weight. Gastric mucosal pH ranged from 1.0 to 2.0 and did not differ between the groups. Microscopic analysis did not reveal any differences in architecture between the two strains (Fig. 5.1A–D) or any differences between genders. The mucosa was of similar thickness with a paucity of intramucosal and submucosal inflammatory cells. Numbers of parietal cells and their distribution within the fundus were similar between groups (WT, 16±1; T-bet, 15±2 parietal cells/gland). A substantial portion of epithelial cell loss and parietal cell apoptosis that occurs during *Helicobacter* infection has been attributed to Fas Ag/FasL signaling (81, 84). Fas Ag is normally expressed at very low levels in gastric mucosal cells, and expression is preferentially up regulated in the parietal and chief cells in the presence of proinflammatory cytokines IL1-β and TNF-α (85). Invading inflammatory cells express both FasL and Fas Ag when activated, and FasL is necessary for Fas-mediated apoptosis of gastric mucosal cells (89). Therefore, to begin our characterization of the T-bet KO mice, we characterized the baseline Fas Ag and FasL expression pattern

FIGURE 5.1 HISTOLOGY OF UNINFECTED WILDTYPE AND T-BET KNOCKOUT MICE ARE SIMILAR.

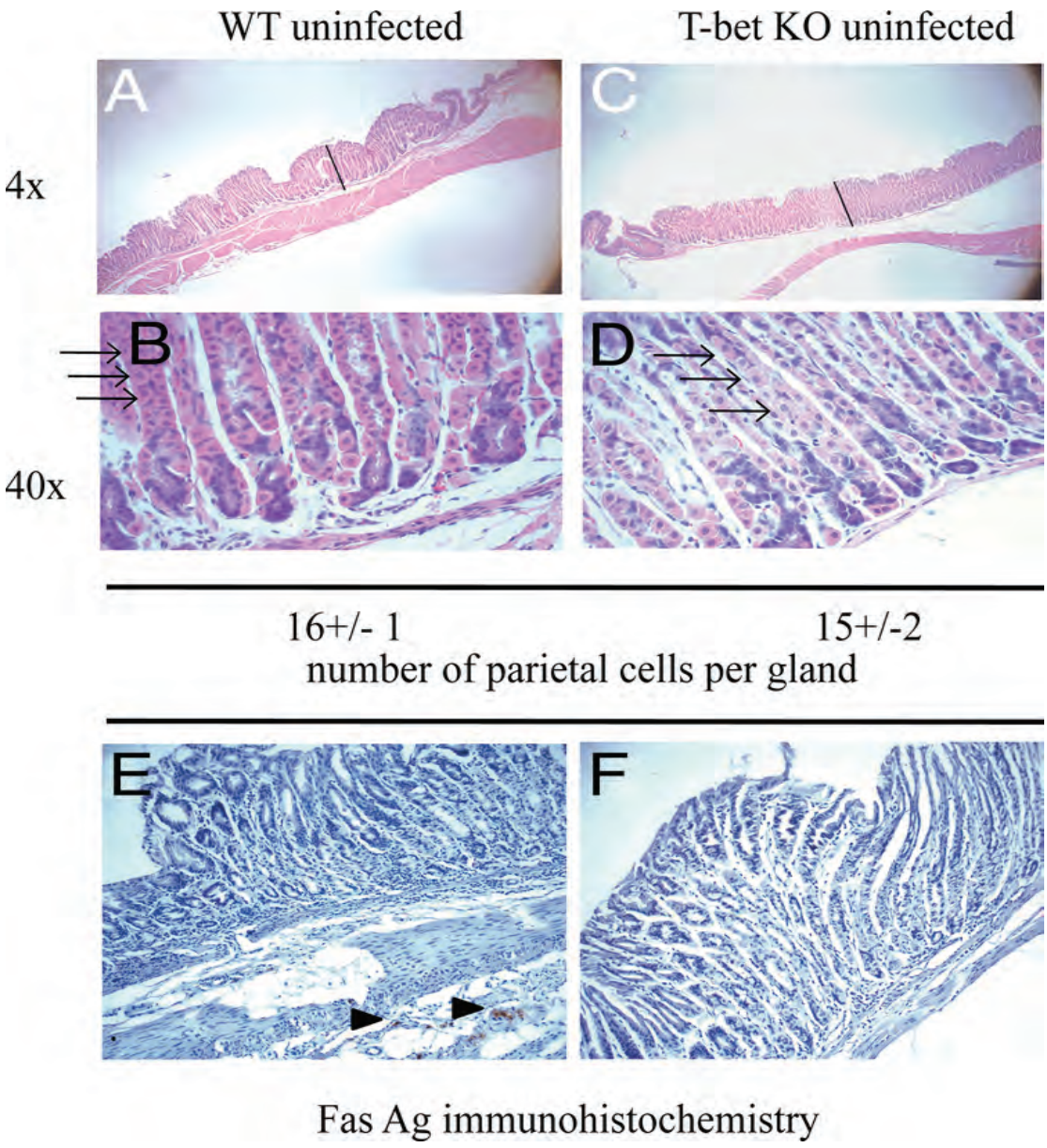


FIGURE 5.1 HISTOLOGY OF UNINFECTED WILDTYPE AND T-BET KNOCKOUT MICE ARE SIMILAR.

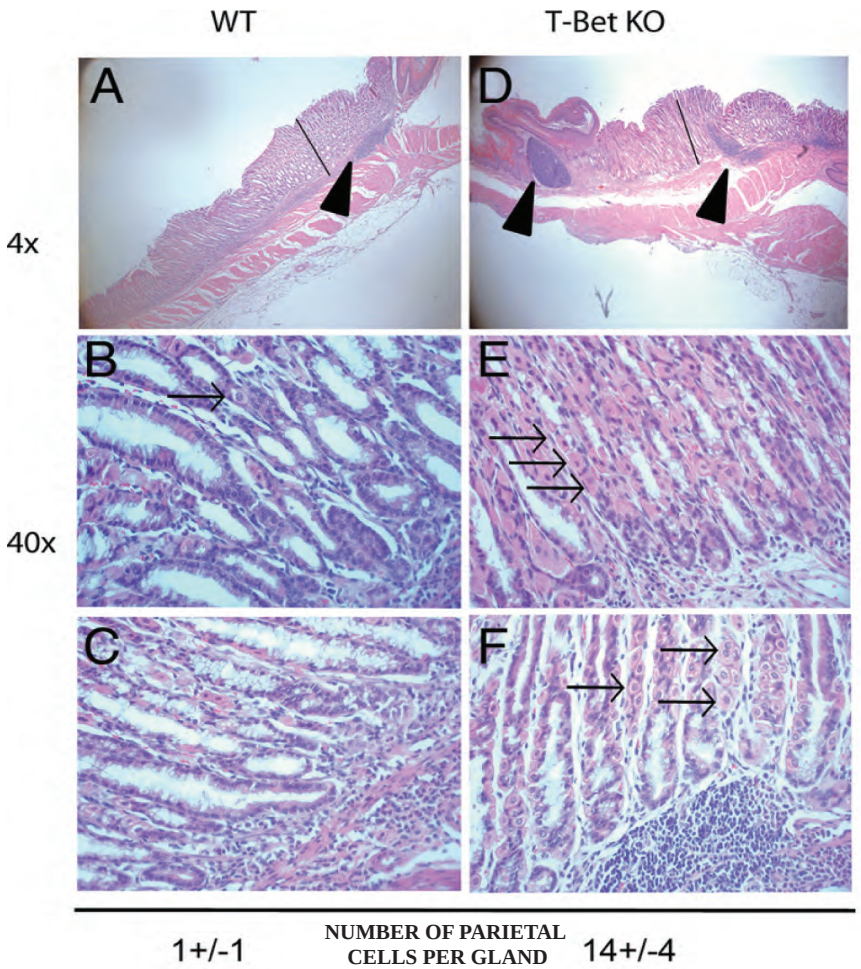
H&E staining of WT C57BL/6 mouse (**A**, X4; **B**, X40) and T-bet KO mouse (**C**, X4; **D**, X40). Parietal cells (arrows) are large cells with pale pink cytoplasm and centrally located nuclei. Chief cells stain deeper purple and are seen at the gland base. Architecture is unchanged between the WT and T-bet KO mouse. Bar, 600 μm. Fas Ag immunohistochemistry in **(E)** WT mouse and **(F)** T-bet KO mouse. Arrow, positive staining inflammatory cells in connective tissue.

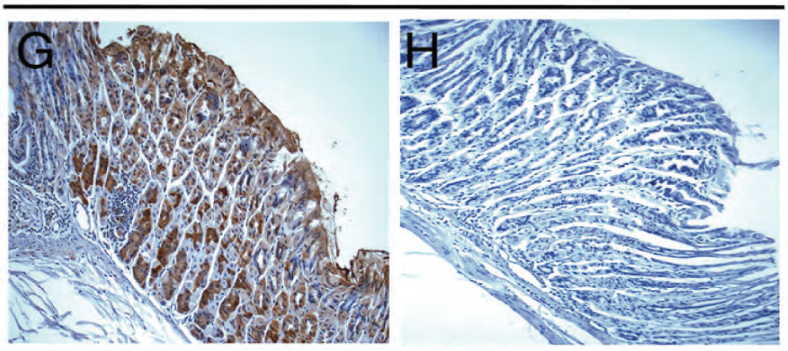
within the gastric mucosa and inflammatory cell population. At baseline, in the absence of infection, Fas Ag is not detectable in gastric mucosal cells of either the WT or the T-bet KO mice (Fig. 5.1, E and F). FasL was not detectable within the mucosa or submucosa of either strain (data not shown). Rare inflammatory cells were occasionally positive for Fas Ag (Fig. 5.1E, arrow).

2. T-bet deficiency protects C57BL/6 mice from parietal cell loss by suppressing Th1 immune response.

At 4 mo of infection WT mice (n=10; 5 male, 5 female) have substantial submucosal (Fig. 5.2A, arrow) and intramucosal inflammatory infiltrates with near complete loss of parietal cells (Fig. 5.2B, arrow shows one remaining parietal cell in this field) and widespread mucous cell hyperplasia (Fig. 5.2C). The gastric mucosal pH ranged from 3 to 7 in the infected WT mice. Like the WT, T-bet KO mice (n=10; 5 male, 5 female) developed widespread inflammatory infiltrates in both the mucosa and submucosa; however, unlike the WT, many T-bet KO mice had large lymphoid aggregates (Fig. 5.2D, arrows). The mucosal height did not differ between WT-infected and T-bet KO-infected mice (Fig. 5.2, A and D, bars). Remarkably, despite substantial inflammation, both male and female T-bet mice maintained their parietal cell population (Fig. 5.2, E and F, arrows) even in areas directly infiltrated with inflammatory cells (Fig. 5.2F), and they maintained a gastric mucosal pH of 1–2. Studies from our laboratory and others reveal that Fas Ag-mediated apoptosis is responsible for the parietal cell dropout seen with *Helicobacter* infection (81, 84).

FIGURE 5.2 MUCOSAL SPARING IN T-BET KNOCKOUT MICE INFECTED WITH *HELICOBACTER FELIS*.





FAS AG IMMUNOHISTOCHEMISTRY

FIGURE 5.2 MUCOSAL SPARING IN T-BET KNOCKOUT MICE INFECTED WITH *HELICOBACTER FELIS*.

H&E staining of the fundic mucosa. Wild type (WT) mouse infected for 4 mo: **(A)** X4, arrow head depicts submucosal inflammation; **(B)** X40, arrow shows one rare remaining parietal amid markedly distorted gland architecture; **(C)** X40, submucosal and mucosal inflammation, mucous metaplasia, and antralization of the glands. T-bet KO infected mouse for 4 mo: **(D)** X4, large intramucosal and submucosal inflammatory infiltrates and lymphoid aggregates (arrow head); **(E)** preservation of architecture with numerous parietal cells (arrows) in areas with intramucosal inflammation; and **(F)** submucosal inflammation. Fas Ag immunohistochemistry of the fundic mucosa at 8 wk of infection in **(G)** WT mouse and **(H)** T-bet KO mouse. Bar, 600 μm.

Therefore, we next determined the regulation Fas Ag expression in the mucosa with infection. Examination of a 2-months time point of infection, when parietal cells are present, revealed intense staining for Fas Ag throughout the parietal and chief cell population of the WT mice (Fig. 5.2G), but not in the T-bet KO mice (Fig. 5.2H). Few remaining parietal cells could be identified in the WT infected mouse at 4 months. Those present expressed surface Fas Ag by immunohistochemistry (data not shown), consistent with our previous reports of Fas Ag/FasL signaling playing a role in parietal cell loss during infection. FasL expression in inflammatory cells within the gastric mucosa did not differ between the WT and T-bet KO mice (data not shown).

3. WT and T-bet KO mice maintain similar levels of H. felis bacterial colonization but have markedly different immunological responses to bacteria.

We next examined *H. felis* bacterial colonization to determine whether differences in infection could account for the disparate mucosal damage seen. Uninfected mice did not have evidence of infection verifying their status as *Helicobacter*-free controls. Infected WT and T-bet mice had similar levels of bacteria detected by PCR, which were not statistically different (Fig. 5.3A; $p>0.05$). Despite similar bacterial loads, the immune response differed substantially with infected T-bet KO mice producing virtually no *Helicobacter*-specific IgG2c, resulting in a markedly blunted IgG2C/IgG1 ratio compared with WT mice, which had developed anti-*Helicobacter* IgG2c Ab (Fig. 5.3B, and C). We did not detect any differences in isotypic responses between male and female mice in either group, and therefore we combined the data for analysis. The gastric mucosa is

FIGURE 5.3 ALTERED IMMUNE RESPONSE IN T-BET KNOCKOUT MICE INFECTED WITH *HELICOBACTER FELIS*.

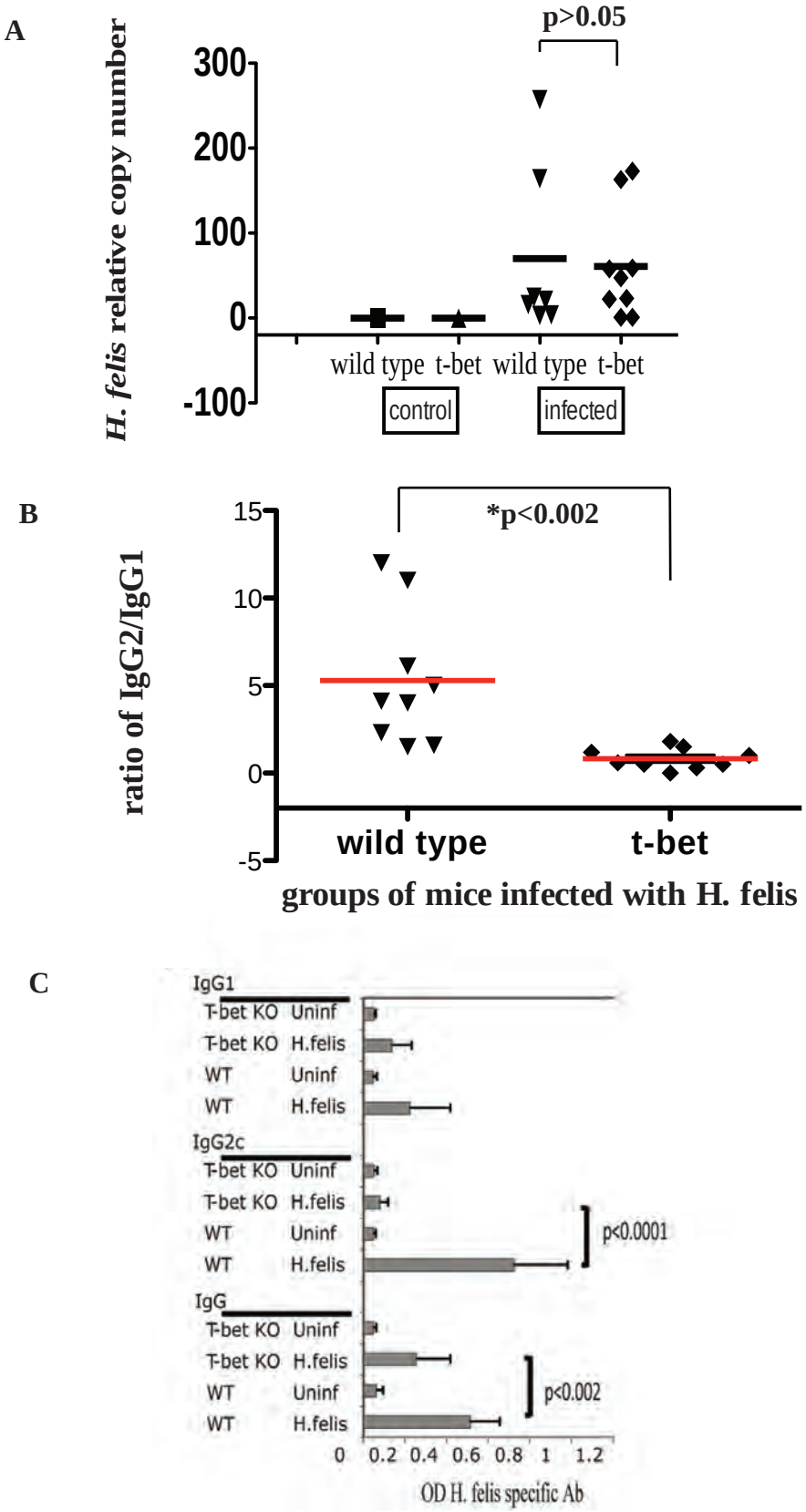


FIGURE 5.3 ALTERED IMMUNE RESPONSE IN T-BET KNOCKOUT MICE INFECTED WITH *HELICOBACTER FELIS*.

(A) Bacterial load in WT (■) and T-bet KO (▲) mice and their infected counterparts WT + *H. felis* (▼) and T-bet KO + *H. felis* (♦) reported as the relative gene expression of *H. felis* FlaB, calculated using the $2^{-\Delta\Delta Ct}$ method and reported as a ratio of fold increase in gene expression over the control value mouse β-actin gene, with uninfected mice set as a value of 1. **(B)** Serum anti-*H. felis* antibodies assay. IgG2C/IgG1 ratio in infected T-bet KO (▼) and WT (◆) mice. **(C)** IgG subclass analysis.

normally devoid of inflammatory cells. Any inflammatory cells within the gastric mucosa must come from circulating sources. We therefore analyzed Fas Ag and FasL expression on splenocytes from WT and T-bet KO mice. With activation, T-bet mice had a slight increase in Fas Ag expression (Fig. 5.4A) and similar FasL expression relative to WT mice (Fig. 5.4B). As predicted, T-bet mice compared with WT mice had a marked blunting of IFN-γ, with both a decrease in the number of cells positive for IFN-γ and the amount of IFN-γ secreted despite similar levels of activation (measured by CD69 expression) (Fig. 5.4, C-E) and similar ratios of CD4 T cells (Fig. 5.4F).

The cytokine milieu of the infected gastric mucosa is an amalgamation of cytokines from innate, adaptive, and mucosal responses. To assess the total cytokine environment, gastric mucosal strips from the squamocolumnar junction through the antrum at the lesser curvature were assayed by RT-PCR for select cytokines. The most dramatic differences between WT and T-bet KO mice were see in TNF-α, IL-1β, IL-12, and IL-13 expression, where the T-bet KO had markedly reduced levels, and in IL-10, where the T-bet KO mice had significantly increased levels relative to the WT mice. While IFN-γ was significantly decreased in the infected Tbet KO mice relative to the WT mice, substantial amounts of IFN-γ were still present (Fig. 5.5 A and B). IL-4 and IL-5 levels were low in both the WT and T-bet KO mice and were not altered with infection.

4. T-bet KO is protective against gastric cancer.

We next evaluated the long-term effects of infection in the WT and T-bet KO

FIGURE 5.4 CHARACTERISTICS OF SPLENOCYTES FROM WILD TYPE AND T-BET KNOCKOUT MICE.

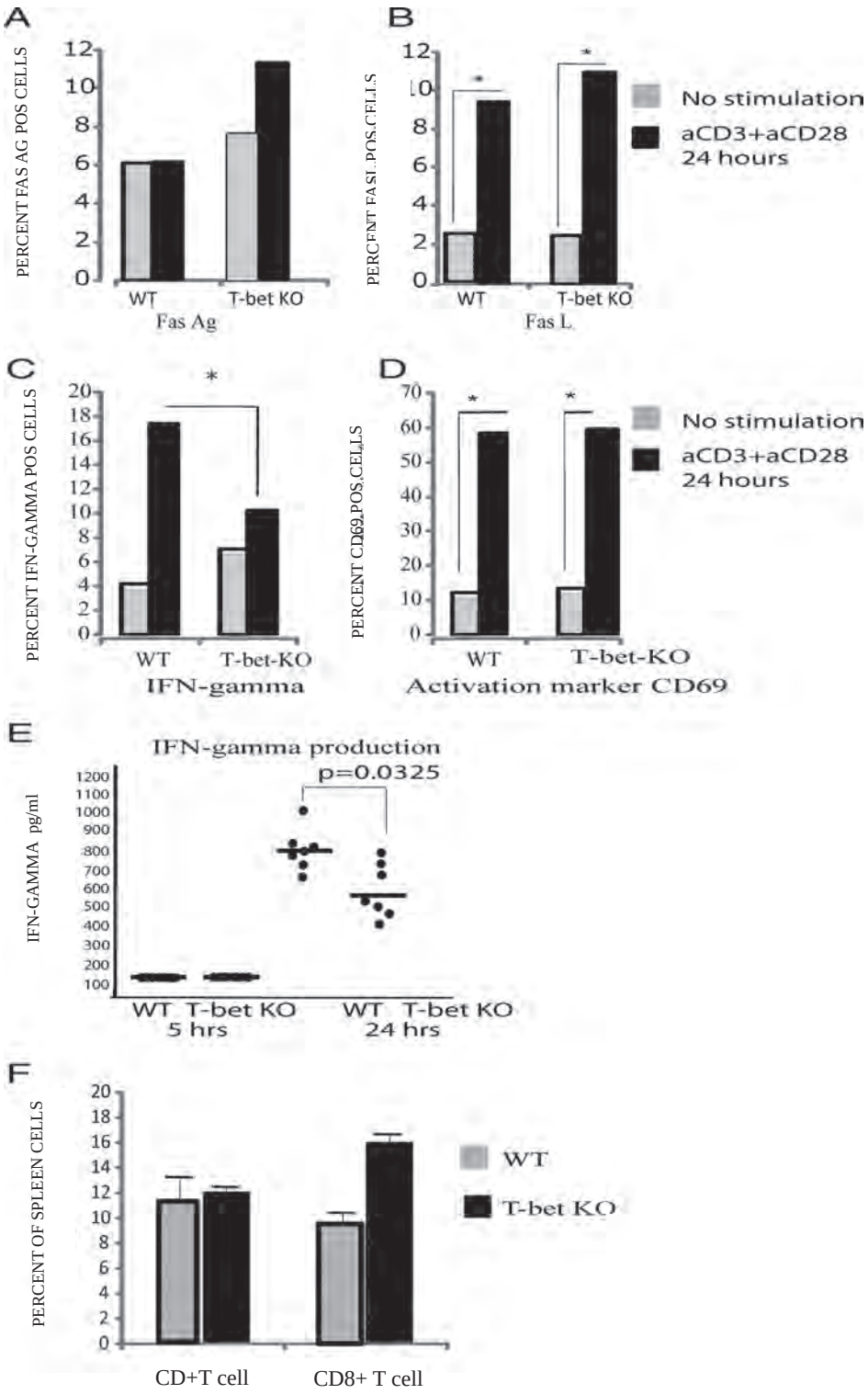


FIGURE 5.4 CHARACTERISTICS OF SPLENOCYTES FROM WILD TYPE AND T-BET KNOCKOUT MICE.

$CD4^+$ cells were activated with anti-CD3 and anti-CD28 as indicated and assayed for **(A)** Fas Ag, **(B)** FasL, **(C)** IFN-γ expression, and **(D)** the activation marker CD69. **(E)** IFN-γ production was measured at 5 and 24 h of activation with anti-CD3 and anti-CD28 in culture. **(F)** The percentage of $CD4^+$ cells did not differ between the WT and T-bet KO mice.

FIGURE 5.5 GASTRIC MUCOSAL CYTOKINE MILIEU IN WILD TYPE AND T-BET KNOCKOUT MICE.

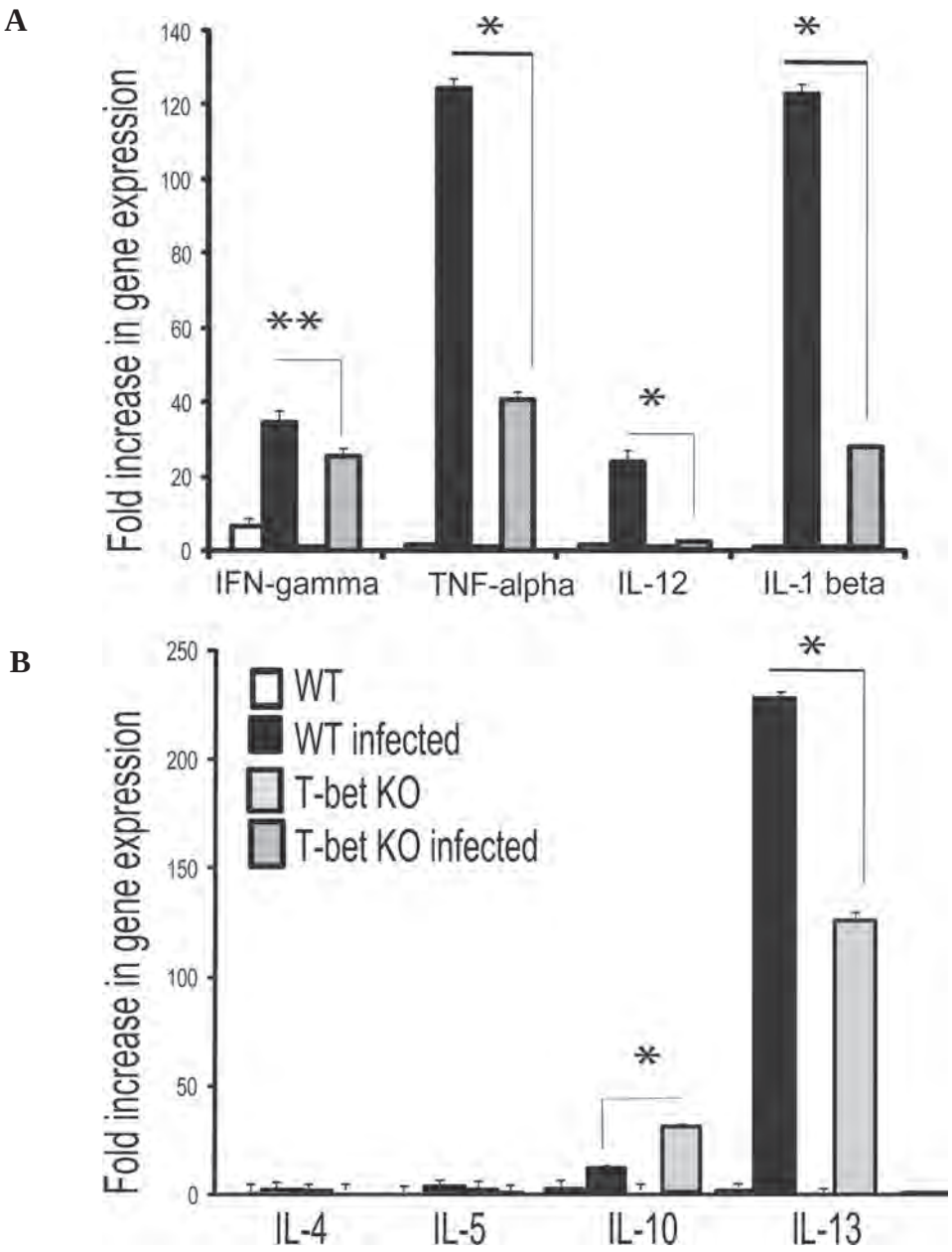


FIGURE 5.5 GASTRIC MUCOSAL CYTOKINE MILIEU IN WILD TYPE AND T-BET KNOCKOUT MICE.

At 4 months after *H. felis* infection, gastric mucosa was assayed for cytokine expression as indicated and reported as fold increase over the lowest reported value in control mice. * $p < 0.001$; ** $p < 0.02$.

mice. *H. felis* bacteria were detected by PCR in all mice, confirming that long-term infection was maintained in both genotypes.

Inflammatory infiltrates were present in both groups, but they differed markedly, with WT mice maintaining intramucosal and submucosal diffuse infiltrates, and T-bet KO mice persisting with prominent lymphoid aggregates in the mucosa and submucosa. No mice developed MALT lymphoma. WT mice progressed through stages of metaplasia, dysplasia, and carcinoma in situ as previously documented (159) over a course of 15 months (Fig. 5.6, A and B). The gastric pH of the infected WT mice ranged from 4 to 7. T-bet KO mice maintained parietal cell populations and gastric pH of 2 or lower in all mice evaluated. They maintained architectural integrity, with only occasional metaplastic glands noted. Infected T-bet KO mice did not develop dysplasia or carcinoma in situ up to 15 mo of observed infection (Fig. 5.6, C and D; and summarized in Fig. 5.6E).

Summary

Our WT and T-bet KO mouse models of *Helicobacter* infection recapitulate the cytokine environment seen in patients who are susceptible to the carcinogenic effects of *Helicobacter* infection (WT) and are apparently resistant to the carcinogenic effects of *Helicobacter* infection (T-bet KO). The striking aspect of this model is the composite mucosal cytokine milieu, contributed to by gastric mucosal cells, macrophages, $CD8^+$T lymphocytes, and other resident cells. Together, these cells produce a cytokine

FIGURE 5.6 INFECTED WILD TYPE MICE DEVELOP GASTROINTESTINAL INTRAEPITHELIAL NEOPLASIA (GIN) AT 15 MONTHS, WHILE T-BET KNOCKOUT MICE ARE PROTECTED.

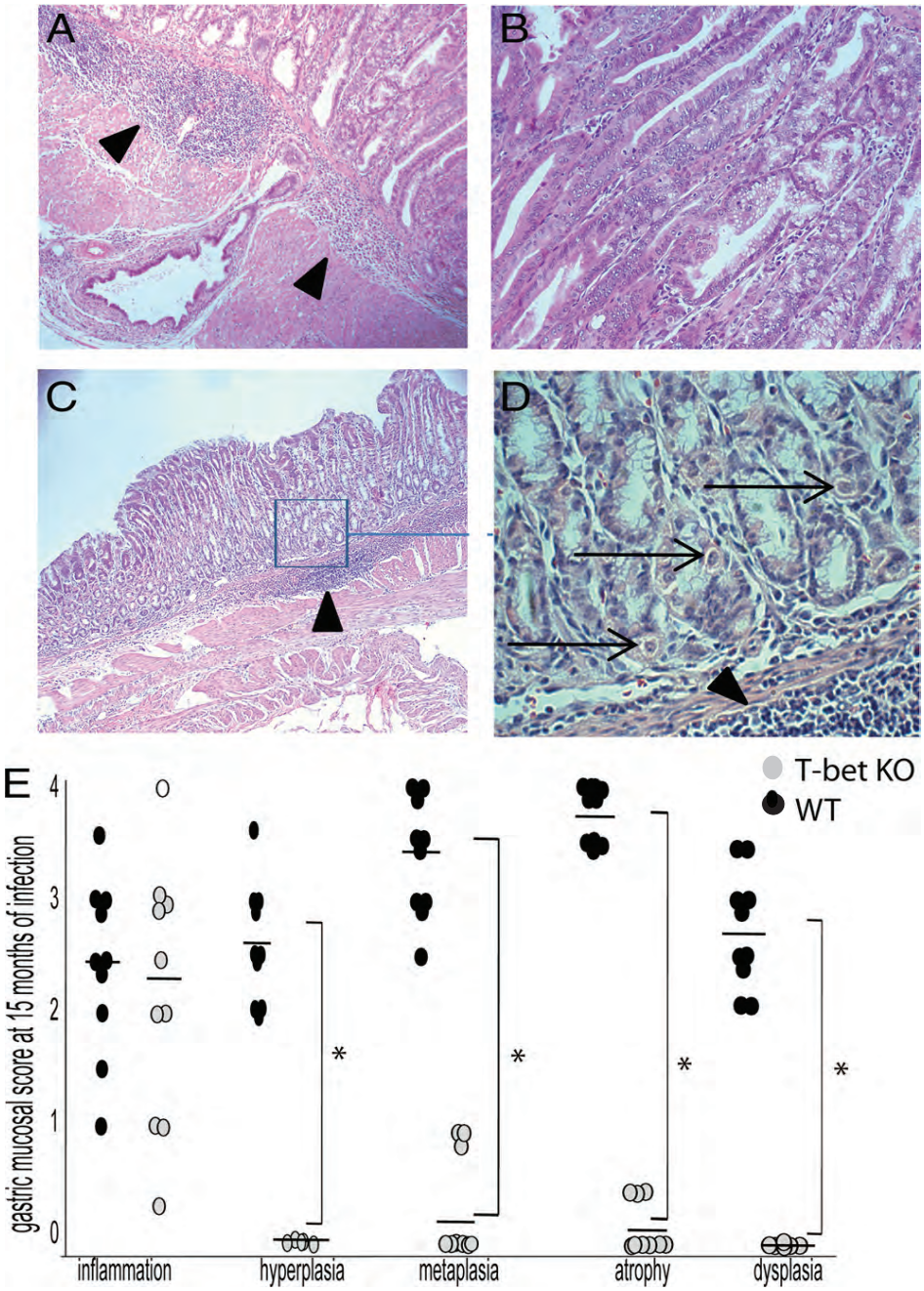


FIGURE 5.6 INFECTED WILD TYPE MICE DEVELOP GASTROINTESTINAL INTRAEPITHELIAL NEOPLASIA (GIN) AT 15 MONTHS, WHILE T-BET KNOCKOUT MICE ARE PROTECTED.

Wild type mice and T-bet KO mice were infected with *H. felis* for 15 months. **(A)** WT mice have persistent inflammation, glandular distortion with marked corpus metaplasia, parietal cell and chief cell loss, and GIN (X4). Gastric glands are seen herniating into the underlying tissue (arrow heads). **(B)** Severe gland distortion with branching, piling up of cells, marked cellular and nuclear atypia with nuclear stratification, and altered nuclear-to-cytoplasmic ratio (X40). **(C)** T-bet KO mice have persistent inflammation (arrowheads) with sparing of parietal (arrows) and chief cell populations (X4); better seen in **D** (X60) of the boxed area in **C**. **(E)** Gastric injury scores are shown below the histology images. * $p < 0.02$.

environment that is difficult to predict based on the functions of T-bet alone. IFN-γ, while lower in the T-bet KO model, was still present at substantial levels within the mucosa, which was a surprising result.

IFN-γ has been associated with parietal cell loss and mucous cell metaplasia in vivo (64, 160), but it is not significantly associated with Fas Ag regulation in vitro (85). Since Fas Ag mediated loss of parietal cells precedes metaplasia in the C57BL/6 model, this implies that IFN-γ may not be acting directly in vivo, and reinterpretation of these models may be needed. We suggest that TNF-α and IL-1β, which do directly regulate Fas Ag, are responsible for Fas Ag up-regulation and parietal cell loss in WT but not T-bet KO mice. IL-4 and IL-5 production are inhibited by T-bet through both an INF-γ-dependent and -independent mechanism (158, 161, 162) and one might expect increased levels of these two cytokines without T-bet; however, the composite mucosal environment does not reflect this, as neither cytokine was present at significant levels. Additionally, IL-13, which can be down-regulated by T-bet, independent of its effect on Th1/Th2 switch (163) would be expected to increase in the T-bet KO mouse; however, a significant paradoxical decrease in IL-13 was seen.

Perhaps the most striking aspect of the gastric mucosal cytokine environment is the dramatic reduction of TNF-α and IL-1β levels in the face of architectural preservation. These two cytokines to date have shown the strongest and most consistent correlation with human *H. pylori*-induced gastric cancer risk, as well as risk for developing premalignant architectural changes (67, 68). Our working hypothesis is that the T-bet mediated immune response to *Helicobacter* results in elevated gastric mucosal

levels of IL-1β and TNF-α. IL-1β and TNF-α in turn mediate regulation and expression of cell surface Fas Ag and subsequent apoptosis of chief and parietal cells (81, 84, 85). Parietal cell loss is associated with decreased gastric acid secretion, which in turn may result in bacterial overgrowth of bowel flora in the stomach (164), although not all studies have confirmed this bacterial overgrowth (165). If present, bacterial overgrowth can further contribute to the antigenic stimulation within the stomach. Fas Ag - mediated signaling plays a central role in the development of gastric cancer through several mechanisms. Architectural changes result in cell-cell signaling defects (64, 156, 160), which are linked to altered differentiation and deregulated proliferation of gastric cells (220). Additionally, the chronic inflammatory environment favors the emergence of apoptotic resistant cell lineages (80, 87, 166-169).

Fas Ag signaling in apoptotic resistant metaplastic and dysplastic gastric cells is diverted toward proliferative signaling via activation of Erk1/2-dependent pathways (91), thus allowing Fas Ag to act as a tumor promoter. T-bet orchestrates this complex immune environment. Knockout of this one critical immune regulator dramatically impacts all the pertinent signaling pathways associated with gastric cancer, suggesting that functional differences in T-bet activity may underlie human susceptibility to gastric cancer.

In previously reported studies (170), T-bet KO mice infected with *H. pylori* did not develop gastric inflammation during the 24 wk of study, and they failed to develop any mucosal alterations, while transfer of $CD4^+$ T-bet KO splenocytes into infected SCID recipients produced significant mucosal damage, similar to transfer of $CD4^+$ WT splenocytes (59). *H. pylori* induces a less robust infection in mice than does *H. felis*, and

it may account for the minimal inflammation noted in the WT mice and the lack of inflammation in the infected T-bet KO mice. SCID mice lack functional B and T cells. Transfer of T-bet KO $CD4^+$ cells would replete only a subset of immune cells in the SCID model, which likely accounts for the differences in overall immune response and mucosal damage between these studies and our results, reported herein.

Many of the severe outcomes of infectious diseases are a direct result of excessive or polarized immune activation. In the case of some pathogens, such as *H. pylori*, it is the immune reaction rather than the pathogen itself that is responsible for the majority of the disease process. In other situations such as inflammatory bowel disease, the inciting pathogen is not known but is postulated to be normal commensal bacteria that are eliciting an abnormal immune response. It is the immune response that leads to ulceration, fibrosis, and predisposition to cancer.

More work has been done examining immune regulation within the small intestine and colon than has been done with the stomach, and therefore drawing from this knowledge may prove useful to advance our understanding of events in the stomach. Within the colon, T-bet has been identified as a master regulator of mucosal inflammation, and indeed our understanding of the role for T-bet in the colon has provided some understanding of perplexing inflammatory conditions. Inflammation within the intestine is normally tightly regulated; however, in a collection of diseases termed inflammatory bowel disease, this regulation is faulty and a chronic inflammatory state is perpetuated. While we think that inflammation is in response to luminal trigger, possibly bacterial, viral, or auto-antigens, the exact trigger has not been determined and is likely not the same in all patients. Animal models of disease have been instrumental in

deciphering the alterations in mucosal immunity and have demonstrated that T-bet activity is pivotal in regulating Th1 predominant Crohn's disease and Th2 predominant ulcerative colitis, where it acts to determine the balance of mucosal cytokines (171). These mouse models are very interesting because the mechanism of dysregulation of T-bet (up-regulation or down-regulation of activity) leads to vastly different cytokine environments and mucosal damage patterns.

Despite the substantial cytokine differences and mucosal outcomes in the mouse model, the point of alteration is identical in both models of inflammatory bowel disease offering a potential clinically relevant target for correction of disease. Inflammatory diseases of the stomach share similarities with inflammatory diseases of the small intestine and colon. Infection with *H. pylori* is associated with a mild asymptomatic gastritis in most patients. Approximately 20% of those infected will develop gastroduodenal ulcer disease while significantly fewer (0.01–3%) will develop gastric adenocarcinoma (154, 155), and even fewer will develop MALT lymphoma. Similar to inflammatory bowel disease caused by opposite immune response in humans, gastric cancer secondary to *H. pylori* infection is associated with opposite immune responses; adenocarcinoma is linked to Th1 cytokines, and MALT lymphoma is linked to Th2 cytokines in people. The widely varying prevalence of gastric adenocarcinoma between patient populations has been attributed to differences in bacterial strains, dietary and environmental cofactors (172), and genetic differences dictating the intensity of the Th1 cytokine response (67, 68). To date, these genetic differences in cytokine response have been investigated at the level of individual cytokine regulation. Work in mouse models using a variety of techniques including infection in Th1 predominant (C57BL/6) and Th2

predominant (BALB/c) strains, manipulation of immune responses through concurrent infections, and use of various cytokine knockout and transgenic models fully support a dependence on a Th1 immune response for adenocarcinoma; however, the dominant cytokine responsible is not clear. Manipulation of individual cytokines such as IFN-γ (64, 160) and IL-1β (173) creates artificial immune environments that result in mucosal damage and share several features of clinical disease, but lack others, suggesting that there are pieces of the puzzle that we still need to fit in place.

In a Chinese population study, *Schistosoma japonicum* infection concurrent with *H. pylori* infection is associated with alterations in IgG responses to *H. pylori* along with less gastric atrophy, suggesting that coinfection alters the immune response of the host to a more favorable outcome (174). Additionally, several African populations have paradoxically low gastric cancer rates despite high infection rates with virulent strains of *H. pylori*. Because of these findings, animal studies have been designed in an attempt to recapitulate the coinfection status of human populations. For example, when the C57BL/6 susceptible strain of mice were infected with both a helminth and *Helicobacter*, the specific *Helicobacter* immune response was skewed toward a Th2 response, and mice were seemingly protected from mucosal damage (65). This effect was not sustained, suggesting that continual ongoing infection with Th2 skewing organisms or an inherent propensity to respond to *Helicobacter* infection with a blunted Th1 response would be needed for long-term effects. Also, in our laboratory we attempted to coerce the BALB/c mouse toward a Th1 response with concurrent *Toxoplasma gondii* infection resulted in a similar, but not identical, pattern of mucosal injury when compared with the C57BL/6

mouse (66) and **CHAPTER III**. These studies have raised the question of whether other genetic factors are at play, or if results are related to an incomplete recreation of the cytokine pattern using these artificial experimental models.

The search in humans for a genetic basis of disease has led to some startling and profound findings, which served as the impetus for this study. Population-based studies assessed genetic polymorphisms in cytokine genes in populations at risk for gastric cancer. Polymorphisms resulting in higher levels of the proinflammatory cytokines IL-1β or TNF-α and lower levels of the antiinflammatory IL-10 were strongly associated with a dramatic increase in *H. pylori*-related noncardia gastric cancer (67, 68). There was an additive effect seen with multiple polymorphisms, conveying the highest risk to patients with the most proinflammatory profile. The original landmark studies by El-Omar and colleagues (77,78) analyzed Caucasian populations. Attempts to reproduce these findings in other populations have met with mixed results (175). Although disappointing, this is not at all surprising given the genetic diversity of patients and the varied genetic mechanisms available to modulate the host immune response. The conflicting results in non-Caucasian populations should be viewed as a challenge to broaden our search for additional regulators of the immune response important for gastric inflammatory disease outcome. Our findings strongly support a central role for T-bet regulation of the immune response associated with gastric cancer risk, and differences in T-bet regulation among patients may account for a portion of this risk.

CHAPTER VI. CXCR4/SDF-1 MEDIATED HOMING OF MESENCHYMAL STEM CELLS TO THE INJURED GASTRIC MUCOSA

Introduction

The link between infection, chronic inflammation, and cancer has long been recognized (176), a prime example being infection with *Helicobacter pylori* and gastric cancer (93, 97). Chronic gastric inflammation, which develops as a consequence of *H. pylori*, leads over time to repetitive injury and repair resulting in hyperproliferation, an increased rate of mitotic error, and progression to adenocarcinoma. The same inflammatory environment that favors the development of cancer has also been linked to homing and engraftment in peripheral tissue by bone marrow derived cells (BMDCs).

In the last decade, there are at least 22 publications, which confirm how a bone marrow derived stem cell is responsible not only for marrow reconstitution, but also for nonhematopoietic tissue homing, engraftment and differentiation (76, 77, 177-194). But which cells from bone marrow are able to differentiate across embryonic lineages? BMDCs plasticity experiments were done with whole bone marrow, hematopoietic stem cells (HSC) or mesenchymal stem cells (MSC), or all of the above in combinations. The analysis of the plasticity mechanisms found that differentiation was a more common phenomenon then cellular fusion, even though the cell fusion was not analyzed in all mentioned above publications.

Finally, how do these cells get there? Most of the models where plasticity was analyzed employed a form of injury or inflammation of peripheral tissue (toxins, radiation, infections, organ resection, etc.), which made the case for secreted factors, like SDF-1 (stromal derived factor 1) and SCF (stem cell factor) to have a role in homing,

since bone marrow derived cells reside at a distant site from the periphery. This may occur where tissue injury induces excessive apoptosis that overwhelms or compromises the supply of endogenous tissue stem cells (195). Evidence suggests that BMDCs use CXCR4/SDF-1 axis for cell trafficking (196, 197). Various tissues respond to cellular damage such as irradiation, hypoxia, chronic inflammation or toxic agents by increasing their secretion of SDF-1 (198-200). The known receptor for SDF-1 is CXCR4.

CXCR4, a G-protein-coupled seven span trans-membrane receptor, is expressed on the surface of many stem cells. CXCR4 is responsible for regulating trafficking of normal hematopoietic stem cells and their mobilization and homing to the bone marrow (197, 201, 202). Furthermore, it is thought that CXCR4/SDF-1 is used during embryogenesis for appropriate cell migration (203). It is well known that hematopoietic stem cells (HSC) depend upon an SDF-1 gradient to home back to the marrow cavity for circulation. Indeed, agents used to mobilize bone marrow cells for transplant purposes propose do so by disrupting the CXCR4/SDF-1 axis (204, 205). In a similar manner, successful engraftment of the bone marrow transplant relies heavily on an intact CXCR4/SDF-1 signaling axis. Inflammatory tissues produce elevated levels of SDF-1, which act as the lure for CXCR4-expressing marrow cells to home to these sites (127). CXCR4 signaling occurs by dissociation of the Gβγ subunit from the GTP bound Gα. Gα will inhibit the adenylate cyclase function, therefore resulting in a reduction of cyclic AMP (cAMP) levels (206, 207) when stimulated by SDF-1.

Similar to their non-malignant stem cell counterparts, many cancer cells express CXCR4. Like the hematopoietic stem cells, it is believed that cancer stem cells use the

CXCR4 receptor to mobilize, invade and metastasize (205), further supporting the notion that cancer stem cells derive from a tissue stem cell source and utilize inflammatory environments for growth and movement. If we look closely at the function of the CXCR4 receptor on normal or malignant cells, we see that it is multi-factorial and modulated by several components of the inflammatory environment.

Although the mechanism and extent of subsequent BMDCs differentiation is not established (208), it is clear that engrafting cells rely on external environmental cues for the orderly inactivation of growth programs and progression of appropriate differentiation (76, 77, 209). However, there is little information on the long-term consequences of recruiting pluripotent cells to areas of chronic inflammation where signals for cell growth and differentiation may be altered.

To investigate the role of BMDCs in the metaplasia/dysplasia/carcinoma progression associated with inflammation, we employed models of short-term inflammation and chronic inflammation in the C57BL/6 mouse model. We postulate that CXCR4/SDF-1 axis might be the key mechanism for recruiting the MSC into a chronic inflammation zone, like *Helicobacter* induced mucosal damage of the stomach.

To determine the role of BMDCs in acute inflammation, we used an acute alcohol model. This is clinically relevant and it is easily reproduced in a mouse model. Topical experimental instillation of alcohol into stomach by gavaging induces local damage and inflammation in the first 30 minutes after application. Ethanol directly and dose-dependently impairs the mucosal barrier, induces the release of inflammatory cytokines and vasoactive substances; these lead to ischemia and mucosal damage (210).

Results

1. Engraftment of Rosa26 marrow-derived cells into the mouse stomach after H. felis infection.

To investigate if BMDCs will engraft into the mouse stomach during an *Helicobacter* infection we used lethally eradiated C57BL/6 mice, which were transplanted with bone marrow from C57BL/6JGtrosa26 (ROSA26) transgenic mice, expressing a nonmammalian beta-galactosidase enzyme or from control wild-type littermates. Engraftment of ROSA26 marrow-derived cells was tracked with X-galactosidase (Xgal) staining.

X-gal staining (blue) was not detected in negative control mice: wild-type noninfected mice (Fig. 6.1A) or wild-type infected mice (Fig. 6.1C). Uninfected ROSA26 transplanted mice did not demonstrate any BMDCs engraftment into gastric glands (Fig. 6.1B). Although acute (3 weeks) *H. felis* infection of ROSA 26 mice was associated with intense bone marrow–derived inflammation, it did not produce major architectural destruction and was not sufficient stimulus for stomach repopulation with BMDCs. In this model, gastric mucosal apoptosis increases at 6 to 8 weeks after inoculation (93) and, consistent with this, β-galactosidase–positive (blue-staining) glands appeared after this peak of apoptosis. Looking into the long-term consequences of inflammation, these cells were initially detected at 20 weeks of infection, but their numbers increased dramatically with the length of time of infection, such that 90% of the gastric mucosa at the

FIGURE 6.1 ENGRAFTMENT OF ROSA26 MARROW-DERIVED CELLS INTO THE MOUSE STOMACH AFTER *H. FELIS* INFECTION.

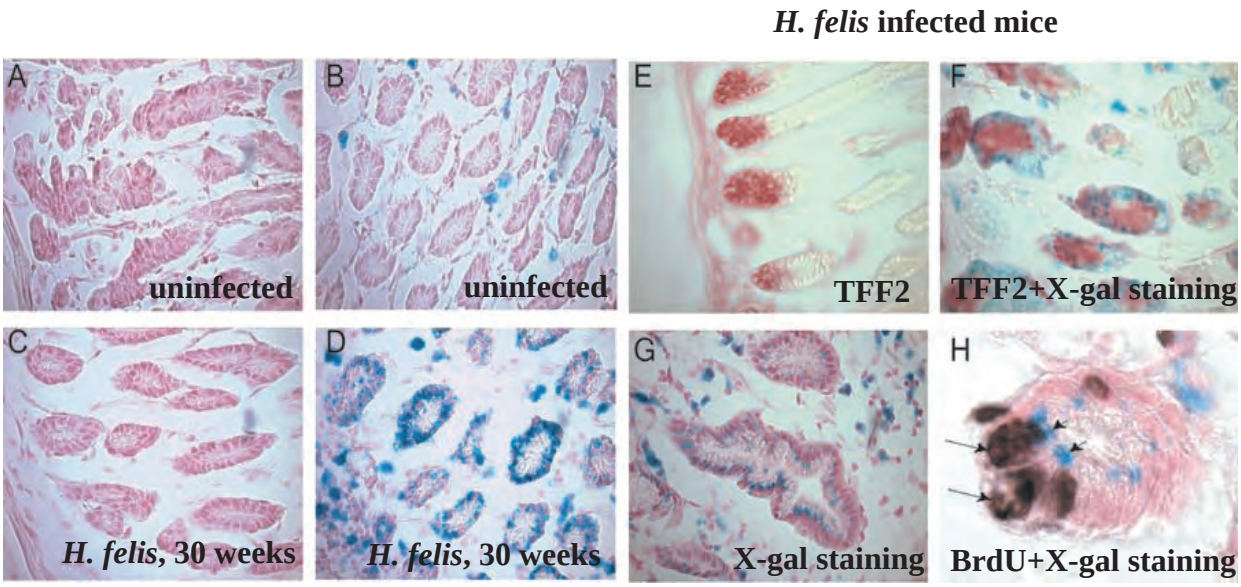


FIGURE 6.1 ENGRAFTMENT OF ROSA26 MARROW-DERIVED CELLS INTO THE MOUSE STOMACH AFTER *H. FELIS* INFECTION.

(A and C) Xgal staining (blue) of C57BL/6 mouse transplanted with wild-type marrow and **(A)** mock infected or **(C)** infected with *H. felis* for 30 weeks. **(B and D)** C57BL/6 mouse transplanted with ROSA26 marrow and **(B)** mock infected or **(D)** infected with *H. felis* for 30 weeks. **(E)** Wild-type mouse with chronic *H. felis* infection shows TFF2 (red) staining and is X-gal negative (blue). **(F)** In the infected ROSA26 transplanted mouse, BMDCs are positive for both beta-galactosidase (blue) and TFF2 (red). **(G)** Dysplastic glands in the infected ROSA26 mouse express abundant beta-galactosidase activity. **(H)** Mitotic activity in BMD epithelial cells demonstrated by coexpression of cytoplasmic beta-galactosidase activity (short arrows; blue) and chromosomal BrdU incorporation (long arrows; brown). 10-mm frozen sections. Magnification: [**(A)** to **(G)**], 60X; **(H)**, 100X.

squamocolumnar junction was replaced with cells derived from donor marrow at 52 weeks after infection (Fig. 6.1D).

With a chronic *Helicobacter* infection, a second proliferative zone forms deeper within the gastric mucosa, giving rise to metaplasia (146). This zone is designated SPEM (spasmolytic expressing metaplasia) because it expresses the trefoil factor 2 (TFF2), also known as spasmolytic polypeptide (211, 212). In chronically infected wild-type mice, TFF2 (red staining) is prominent in deep antral and fundic glands (Fig. 6.1E). In mice transplanted with ROSA26 marrow and infected with *H. felis*, TFF2 expression is seen within blue β-galactosidase–positive BMDCs (Fig. 6.1F). Histological alterations were similar in infected WT and ROSA26-transplanted mice, with both showing equivalent metaplasia and dysplasia. Of the few parietal cells or chief cells that persisted in the infected stomach, none were beta-galactosidase positive, which indicates that under these experimental conditions of *H. felis* infection, marrow cells do not differentiate toward the parietal or chief cell phenotype. Epithelial dysplasia increased in severity over time, and by one year after inoculation resulted in carcinoma or high-grade gastrointestinal intraepithelial neoplasia (GIN) (213). In the mouse model of *Helicobacter* mediated gastric cancer, dysplasia is considered a direct precursor of gastric adenocarcinoma and is found both at the squamocolumnar junction and at the antral-pyloric junction (214, 215). In this *H. felis* model, the majority of dysplastic glands stained blue with X-gal (Fig. 6.1G), and many BMDCs within the epithelium, were bromodeoxyuridine (BrdU) positive (Fig. 6.1H), which demonstrates active proliferation as occurs in neoplasia.

2. β-Galactosidase immunohistochemistry (IHC) and immunofluorescence (IF) of stomachs from C57BL/6 mice transplanted with Rosa26 marrow and infected with H. felis.

To further confirm the presence of β-galactosidase, we used immunohistochemistry (IHC) for bacterial β-galactosidase. Gastric tissue from wild-type mice did not stain for β-galactosidase (Fig. 6.2, A and C), whereas all observed intraepithelial neoplasia in the 52- week infected mice were β-galactosidase positive (Fig. 6.2, B and D; brown intracellular staining), which proves that these cells arose from donor marrow and strongly suggests an inherent vulnerability of this population to malignant progression. Bone marrow–derived GIN displayed features consistent with this histological diagnosis (213), including elongation and branching, crowding and distortion of gland structures, presence of hyperchromatic nuclei, pronounced cellular and nuclear atypia, and loss of polarity.

To further test if the β-galactosidase positive cells are indeed epithelial cells, but not donor bone-marrow derived lymphocytes a double-label immunofluorescence staining revealed that the β-galactosidase–positive cells (red) within deep gastric glands were also pan-cytokeratin positive (green; merged seen as yellow) (Fig. 6.2F and fig. 6.3D). CD45 expression (red; 6.3 J and N) was specifically restricted to donor derived infiltrating leukocytes (red; fig. 6.3K; merged yellow; 6.3L). These studies confirmed that the marrow-derived cells had differentiated to a gastric epithelial phenotype, ruling

FIGURE 6.2 BETA-GALACTOSIDASE IMMUNOHISTOCHEMISTRY (IHC) OF STOMACHS FROM C57BL/6 MICE TRANSPLANTED WITH ROSA26 MARROW.

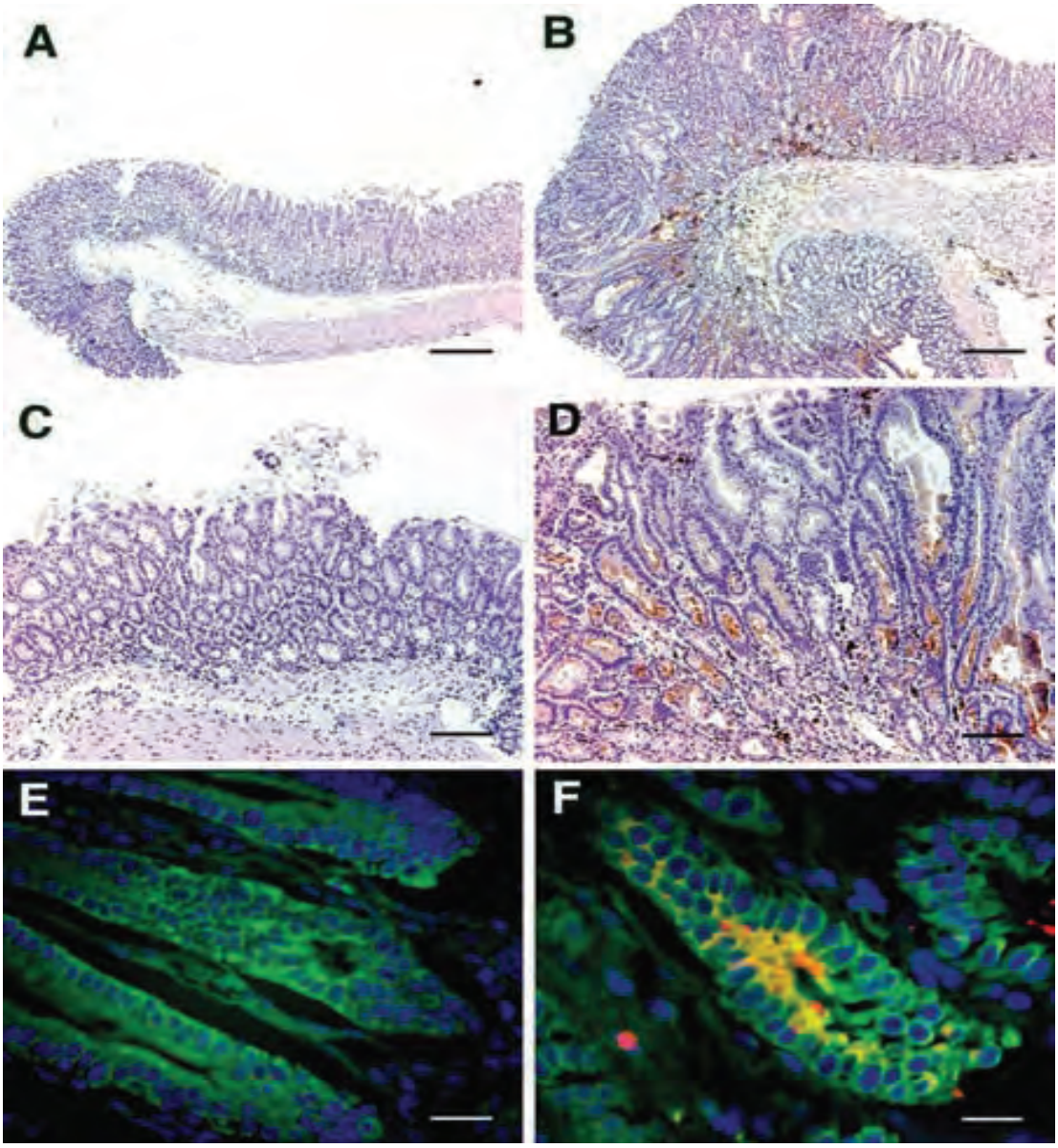


FIGURE 6.2 BETA-GALACTOSIDASE IMMUNOHISTOCHEMISTRY (IHC) OF STOMACHS FROM C57BL/6 MICE TRANSPLANTED WITH ROSA26 MARROW.

(A and **C)** Mock-infected mice do not demonstrate any BMDC engraftment, as evidenced by lack of beta-galactosidase staining. **(B** and **D)** *H. felis* – infected mice have substantial architectural distortion and betagalactosidase – positive (brown) GIN. Fluorescence IHC for cytokeratin (green) and betagalactosidase (red). **(E)** Glands within GIN from an infected mouse transplanted with wildtype marrow do not express betagalactosidase. **(F)** Glands within GIN from an infected mouse transplanted with ROSA26 marrow demonstrate beta-galactosidase expression (red), colocalized with cytokeratin (green) to form yellow, confirming epithelial differentiation of integrated BMDC. Occasional mononuclear leukocytes are betagalactosidase positive (red) and cytokeratin negative. Scale bars, 400 mm [**(A)** and **(B)**], 160 mm [**(C)** and **(D)**], 40 mm [**(E)** and **(F)**].

FIGURE 6.3 IDENTIFICATION OF MARROW DERIVED CELLS IN THE GASTRIC MUCOSA USING IMMUNOFLUORESCENCE (IF).

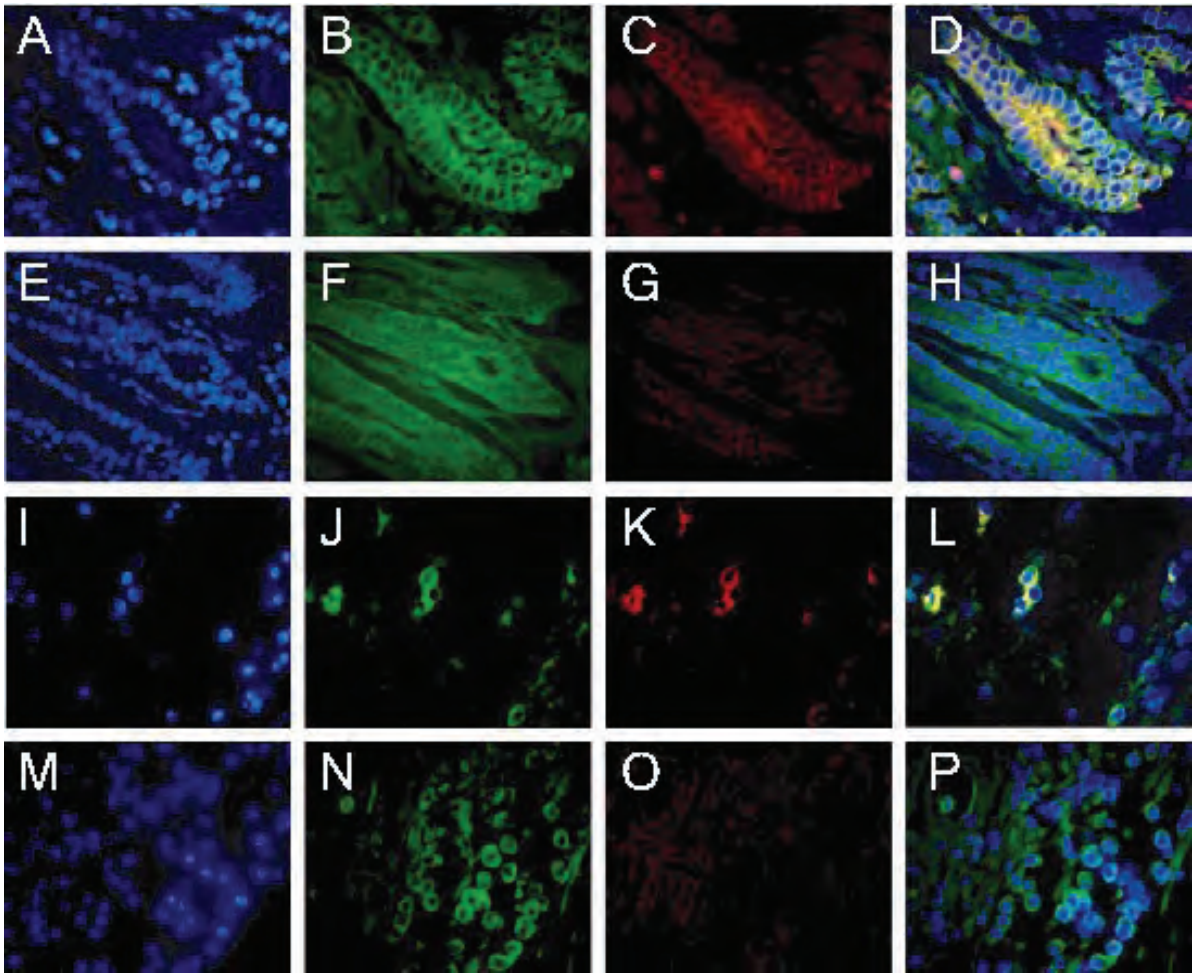


FIGURE 6.3 IDENTIFICATION OF MARROW DERIVED CELLS IN THE GASTRIC MUCOSA USING IMMUNOFLUORESCENSE (IF).

Mice transplanted with ROSA26 **(A-D** and **I-L)** or WT **(E-H** and **M-P)** marrow were infected with *Helicobacter felis* for 52 weeks. Nuclear morphology is similar (DAPI staining, first panel-blue) in all groups. Gastric mucosal cells stain for cytokeratin (second panel- green) in both the ROSA26 **(B)** and the WT **(F)** transplanted mouse. Beta-galactosidase expression (third panel- red) within gastric gland units is only seen in the mouse transplanted with ROSA26 **(C)** and not WT **(G)** marrow. In the merged panels (last column) the mouse transplanted with ROSA26 marrow **(D)** demonstrates colocalization of beta-galactosidase (red) with cytokeratin (green) producing yellow while **(H)**. WT mice do not co-localize signals. Infiltrating inflammatory cells are CD45 positive (green) in mice transplanted with ROSA26 **(J)** or WT **(N)** marrow. Betagalactosidase (red) is only expressed in ROSA26 **(K)** derived leukocytes, and is not found in WT **(O)** transplanted mice. Merged CD45 (green) and beta-galactosidase (red) are seen as yellow **(L)** in the ROSA transplant mice, and this merged signal is absent in WT mice **(P)**.

out the unlikely possibility that the observed staining pattern was due to lymphocytes ($CD45^+$) intercalating into the gland structure.

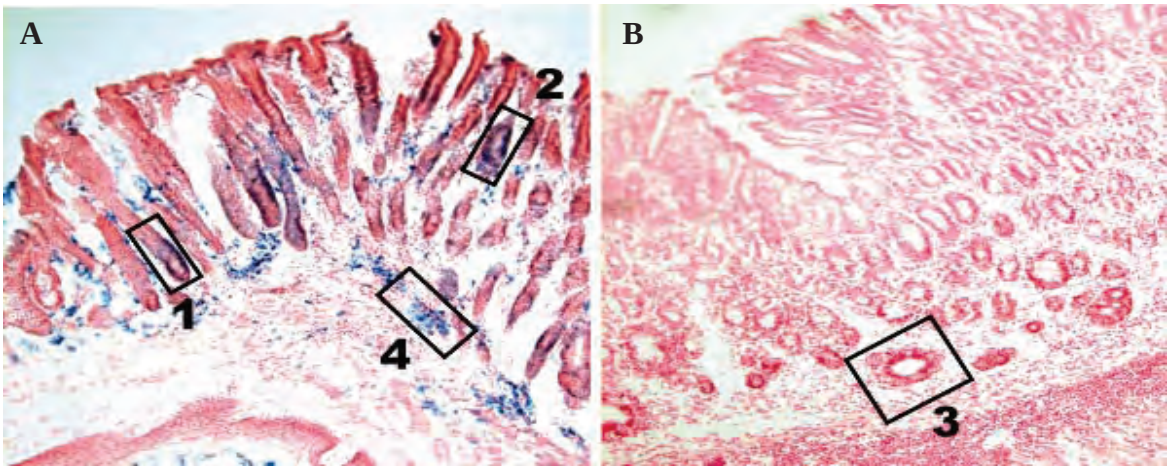
3. Engrafted BMDCs contain the LacZ/Neo fusion gene.

Although these analyses directly showed β-galactosidase enzyme activity (X-gal staining) and protein abundance (IHC), we further evaluated BMDCs within the epithelium for the lacZ/Neo fusion gene specific to donor cells from the ROSA26 mouse. Laser capture microdissection was used to capture and isolate entire X-gal–positive glands from chronically *H. felis*–infected ROSA26 and wild-type transplanted mice (Fig. 6.4, A and B). This was followed by a polymerase chain reaction (PCR) with specific lacZ/Neo fusion gene primers, followed by sequence analysis verified these cells to be of donor origin (Fig. 6.4C, lanes 1, 2 and 4). Water control of the PCR reaction was negative (Fig. 6.4C, lane 5).

4. Gastric mucosal cells sorted and analyzed by flow cytometry.

As a further additional test to prove for bone marrow origin of the gastric epithelial cells, we used a completely independent model of labeled bone marrow reconstitution. Female C57BL/6 mice were lethally irradiated, transplanted with bone marrow from male transgenic mice expressing chicken β-actin-EGFP (enhanced green fluorescent protein), and then infected with *H. felis* for 15 to 16 months. Dispersed gastric

FIGURE 6.4. ENGRAFTED BMDC CONTAIN THE LACZ/NEO FUSION GENE.



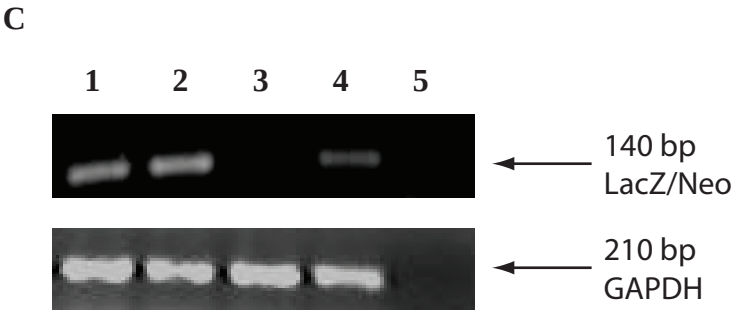


FIGURE 6.4 ENGRAFTED BMDC CONTAIN THE LACZ/NEO FUSION GENE.

Laser capture microdissection was used to isolate beta-galactosidase positive gastric glands **(A**- boxes 1 and 2) or beta-galactosidase positive infiltrating leukocytes (**A**-box 4) from a mouse transplanted with ROSA26 marrow. As a negative control, we used a captured gastric gland from a mouse transplanted with WT marrow (**B**- box 3). **(C)** PCR for the LacZ/Neo fusion gene and GAPDH show that the LacZ/Neo expression is specific to engrafted cells. The numbers correspond to the boxes in panels **(A)** and **(B)**. Lane 5 is a water control.

mucosal cells from these mice were sorted by flow cytometry (Fig. 6.5A) into $GFP^+/CD45^+$ (P4) – donor derived leukocytes, $GFP^+/CD45^-$ (P5) – donor derived epithelial cells, and $GFP^-/CD45^-$ (P6) populations – host derived epithelial cells, subsequently stained for pan-cytokeratin, and also analyzed by fluorescent in situ hybridization (FISH) for X and Y chromosomes. The $CD45^-$ populations were consistently positive for pan-cytokeratin (Fig. 6.5, D and F), which indicates their epithelial nature, whereas $CD45^+$ cells were negative for this marker (Fig. 6.5B) – indicating their nonepithelial lineage. $GFP^-/CD45^-$ cells contained two X chromosomes (Fig. 6.5G), which confirms them to be of host origin, whereas all GFP^+ cells were consistently positive for both X and Y chromosomes by FISH (Fig. 6.5, C and E), which demonstrates that they are of donor bone marrow origin.

5. Tumor cells are GFP positive, Y chromosome positive and coexpress cytokeratin.

Analysis of tissue sections from the female mice transplanted with bone marrow from male transgenic mice expressing chicken β-actin-EGFP (Fig. 6.6) demonstrated that tumor cells were GFP positive (brown stain) (Fig. 6.6B and C) and Y chromosome positive (green signal) and expressed cytokeratin (red signal) (Fig. 6.6E and F). FISH for Y chromosome is negative in the female-to-female transplant animal (cytokeratin; red) (Fig. 6.6D). These studies, using two independent markers (GFP and Y chromosome), in addition to the β-galactosidase marker, confirmed that in *Helicobacter*-infected mice, bone marrow– derived cells could give rise to gastric epithelial cancer.

FIGURE 6.5 GASTRIC MUCOSAL CELLS SORTED AND ANALYZED BY FLOW CYTOMETRY.

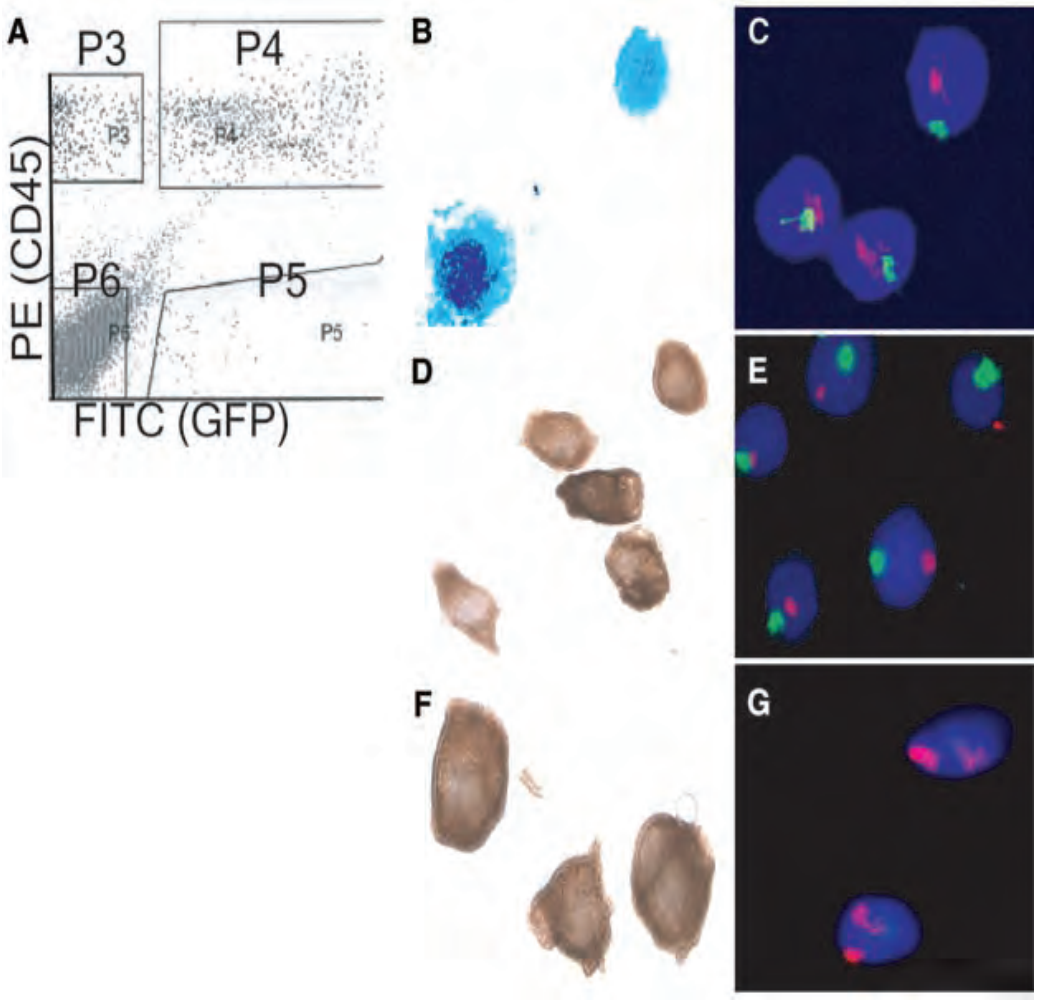


FIGURE 6.5 GASTRIC MUCOSAL CELLS SORTED AND ANALYZED BY FLOW CYTOMETRY (FACS).

(A) FACS sorting of gastric mucosal cells from long-term *H. felis* – infected female mice transplanted with male GFP^+ bone marrow. Three populations were sorted and characterized further. **(B)** The population sorted, as $GFP^+/CD45^+$ (P4) does not stain for cytokeratin (hematoxylin counterstained). **(C)** X (red) and Y (green) FISH confirms that the $GFP^+/CD45^+$ (P4) population is made up of donor-derived leukocytes. **(D)** The population sorted as $GFP^+/CD45^-$ (P5) is made up of donor derived engrafted gastric mucosal cells that stain for cytokeratin (brown) and **(E)** contain both X and Y-chromosomes by FISH. **(F)** $GFP^-/CD45^-$ cells (P6) are cytokeratin positive and **(G)** contain two X chromosomes, confirming that they are host-derived gastric epithelial cells.

FIGURE 6.6 TUMOR CELLS ARE GFP POSITIVE, Y CHROMOSOME POSITIVE AND EXPRESSED CYTOKERATIN.

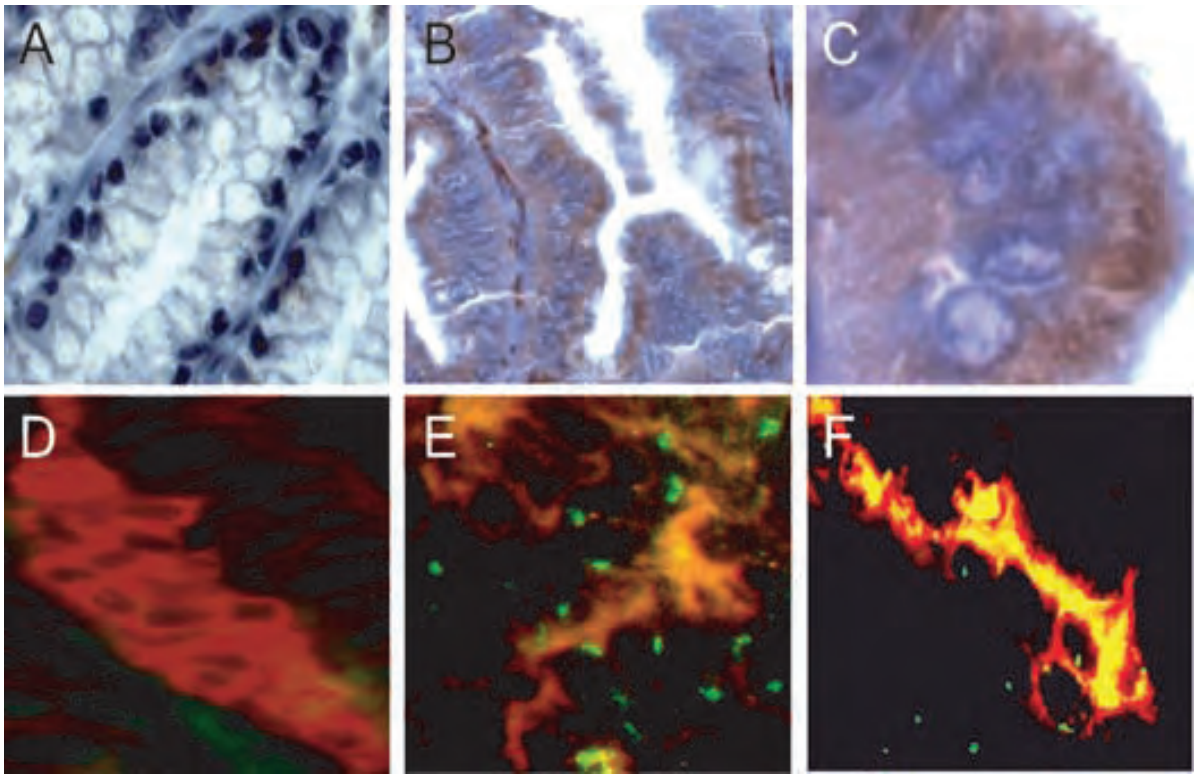


FIGURE 6.6 TUMOR CELLS ARE GFP POSITIVE, Y CHROMOSOME POSITIVE AND EXPRESSED CYTOKERATIN.

(A) Female wild-type mouse transplanted with female wild-type marrow does not stain for GFP by IHC. **(B** and **C)** Female mouse transplanted with male GFP marrow shows positive IHC staining for GFP (brown staining) in tumor cells. **(D)** FISH for Y chromosome (green) is negative in the female-to-female transplant (cytokeratin; red). **(E** and **F)** Tumors from male-to-female transplants show numerous Y-chromosomes (green) within the nuclei (black) of cytokeratin-positive (red) cells.

6. BMDCs did not fuse with gastric epithelium after engraftment.

Cell-to-cell fusion, where an engrafting cell merges with a preexisting differentiated target cell, is another way towards multilineage plasticity. The resulting tetraploid cell could be one nucleated (if nuclear fusion occurred) or binucleated, if it did not. However, a reductive division may then follow, which could result in a mixed nucleus-cytoplasm combination.

Using three methods, we next addressed whether fusion occurred between BMDCs and the gastric epithelium. First, we examined histological sections containing bone marrow–derived epithelial cells and found that cells contained only a single nucleus; no binucleate cells were seen. Second, we used fluorescence activated cell sorting (FACS) of propidium iodide–stained cells to determine DNA content in wild-type tissues, early infection prior to engraftment, and BMDCs carcinoma, and did not demonstrate any difference between these groups (Fig. 6.7). Third, in female mice transplanted with male GFP transgenic marrow, we evaluated 10,000 GFP^+/CD45–pancytokeratin$^+$ FACS-sorted gastric cells, using FISH, and showed a single X and a single Y chromosome in all cells examined (Fig. 6.5E). These studies strongly suggest that stable fusion did not occur. In our initial reconstitution studies, whole bone marrow was used to minimize cell manipulation, which can alter growth potential and behavior of stem cells (77, 208).

FIGURE 6.7 FURTHER EVIDENCE THAT STABLE FUSION IS NOT THE MECHANISM BY WHICH BMDC DIFFERENTIATE TO GASTRIC MUCOSAL CELLS, METAPLASIA, DYSPLASIA OR EARLY CANCER.

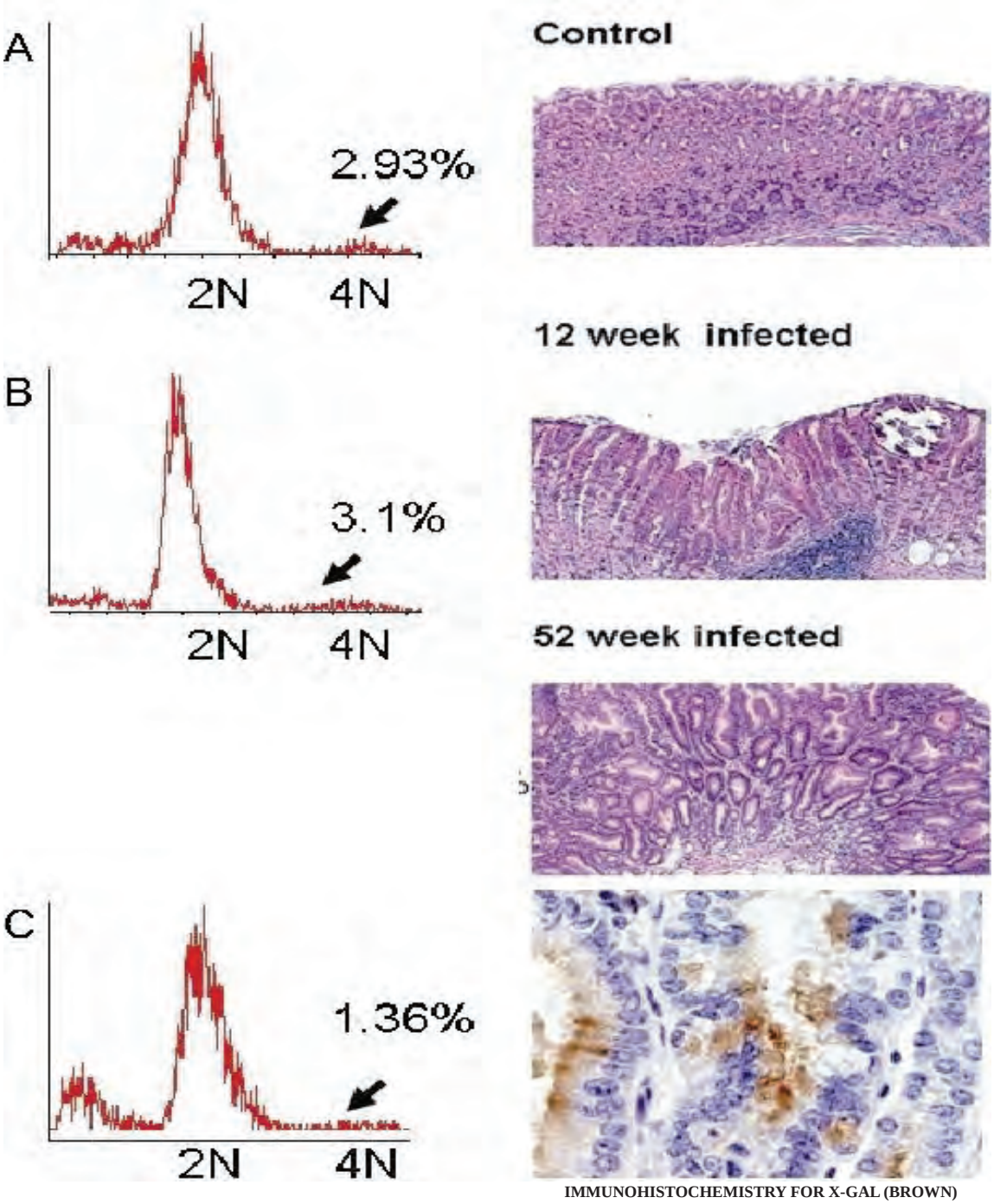


FIGURE 6.7 FURTHER EVIDENCE THAT STABLE FUSION IS NOT THE MECHANISM, BY WHICH BMDC DIFFERENTIATES TO GASTRIC MUCOSAL CELLS, METAPLASIA, DYSPLASIA OR EARLY CANCER.

(A) DNA content and gastric mucosal histology from a one-year-old male WT mouse without *Helicobacter* infection **(B)** WT transplanted with ROSA26 marrow and infected for 12 weeks or **(C)** 52 weeks. Single cell preparations were prepared from the gastric mucosa and analyzed by FACS for DNA content. The number of >2N cells was not different between groups. Histology confirms the presence of a single nucleus in each mucosal cell. In long term infected mice **(C)** GIN is seen in the H&E section, with the majority of mucosa at the squamocolumnar junction along the lesser curvature replaced by BMDC (brown staining- IHC for beta-galactosidase, bottom panel). Only single nuclei are seen in these BMD-gastric epithelial cells.

7. Mesenchymal stem cells, but not the hematopoietic stem cells, acquired a gastric mucosal phenotype when exposed to primary epithelial cell cultures.

To identify the population of cells within the bone marrow responsible for gastric mucosal engraftment, we cultured hematopoietic stem cells (HSC) (lineage depleted, $Rho^{dull}Ho^{dull}$) or adherent mesenchymal stem cell (MSC) populations in Transwell culture plates in contact with the soluble components of control medium or culture medium from primary gastric epithelial cell cultures. Neither HSC nor MSC populations expressed epithelial markers (TFF2 or KRT1-19 – keratin 1-19) at the time of isolation or after culture with control medium. MSC cultures, but not HSC cultures, showed a marked up-regulation of both KRT1-19 and TFF2 at 24 and 48 hours when exposed to the soluble components of gastric epithelial tissue, which demonstrates that MSC (but not HSC) acquired a gastric mucosal cell–gene expression pattern without cell-to-cell contact or fusion (Fig. 6.8).

8. Acute alcohol and H. felis induced inflammation promote a significant upregulation of stromal derived factor 1 (SDF-1) in the stomach.

Next, we wanted to test if the components of the CXCR4/SDF-1 axis have a role to play in the stem cell mobilization towards an inflamed stomach mucosa. To do this we performed real time RT-PCR analysis on whole gastric mucosa from *H. felis*–infected C57BL/6 mice (6 and 12 months after infection), which showed a substantial up-

FIGURE 6.8 THE GASTRIC MUCOSA PROMOTES DIFFERENTIATION OF MESENCHYMAL STEM CELLS TOWARD AN EPITHELIAL CELL PHENOTYPE.

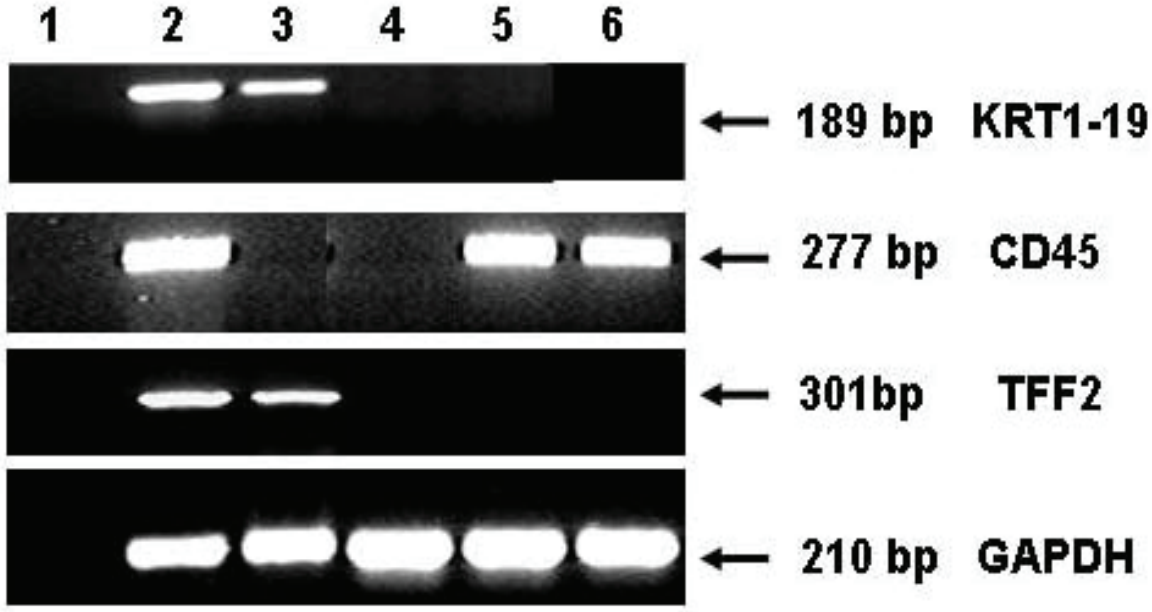


FIGURE 6.8 THE GASTRIC MUCOSA PROMOTES DIFFERENTIATION OF MESENCHYMAL STEM CELLS (MSC) TOWARD AN EPITHELIAL CELL PHENOTYPE.

Hematopoietic stem cells (HSC) or MSC were cultured in control medium or with the soluble components of a primary gastric mucosal cell culture followed by RT-PCR after 48-hours. Lane 1: Negative water control. Lane 2: Positive control- RNA isolated from the gastric mucosa from an infected male mouse KRT1-19 (epithelial cell cytokeratin), CD45 (from infiltrating leukocytes), and TFF2 (metaplastic cell lineage marker) are all expressed. Lane 3: Mesenchymal stem cells cultured in the presence of gastric mucosa do not express CD45 but do express both KRT1-19 and TFF2. MSC do not express these epithelial cell markers under control conditions (lane 4). Lane 5- Lineage depleted Rho^{dull}, Ho^{dull} (HSC) cells express CD45, but do not express KRT1-19 or TFF2 when exposed to gastric mucosal environment or with control medium (lane 6). Loading quantity was standardized with GAPDH.

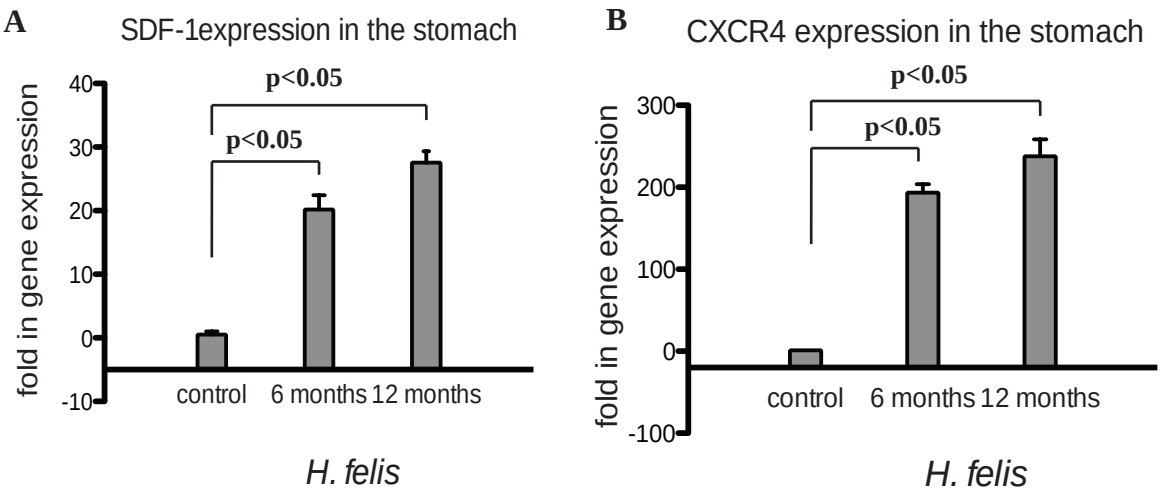
regulation of SDF-1 and CXCR4 compared with uninfected age-matched controls (Fig 6.9A and B). Immunohistochemistry performed on the stomach tissue confirms the presence of CXCR4 on the dysplastic epithelial glands in an infected animal (Fig 6.9 C and D). Recent evidence suggests that SDF-1/CXC4 axis plays an important role not only in mobilization of the bone marrow cells, but also in tumor cell growth, mobilization and metastasis (216-219).

We were also able to confirm the upregulation of these two factors in an acute inflammation model using ethanol. Acute alcohol injury in mice induces up to 9 fold SDF-1 upregulation in the stomach (Fig. 6.10A) and a severe inflammation score (2.5) for epithelial lesion changes, after giving mice 3 doses of alcohol (Fig. 6.10B and D). Histological analysis shows diffuse mucosal ulceration and severe lymphocytic infiltration in the stomach, which looked hemorrhagic on gross examination. Therefore, the acute alcohol injury model could be used to study the homing properties of the BMDCs.

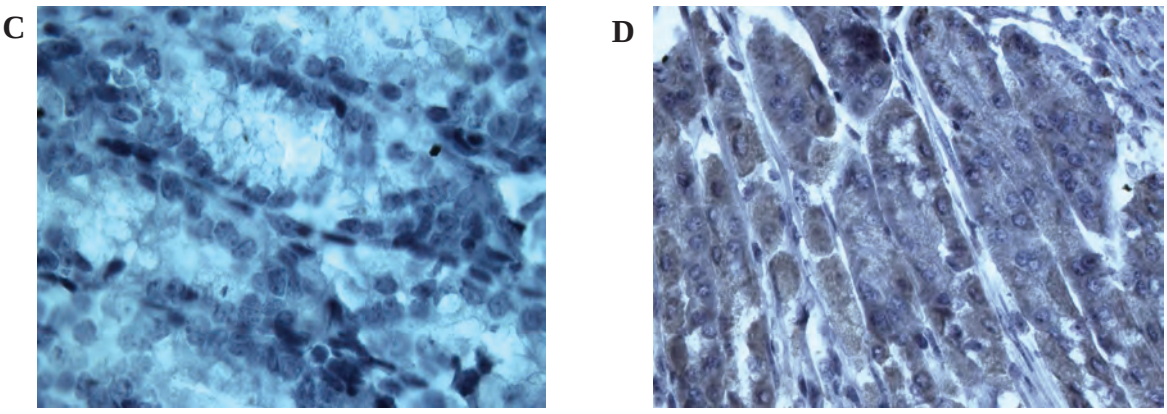
9. Red fluorescent protein (RFP) positive mesenchymal stem cells (MSC) home in the stomach and differentiate into gastric epithelial cells.

Our laboratory had developed several culture adapted mesenchymal stem cell lines from a male C57BL/6J mouse by continuous propagation of its total bone marrow in tissue culture (220). With each passage, cells that were attached to the plate and became confluent were analyzed for their ability to form foci or grow in soft agar, proving if they underwent transformation. FACS analysis of our MSC showed that they were CD44

FIGURE 6.9 SDF-1 AND CXCR4 UPREGULATION IN THE STOMACH AFTER *HELICOBACTER* INFECTION.



IMMUNOHISTOCHEMISTRY FOR CXCR4 IN THE STOMACH



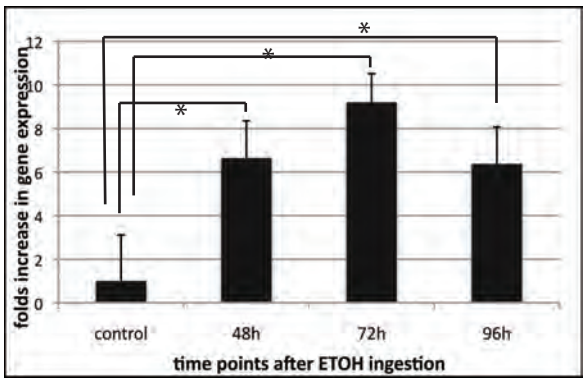
CONTROL, 60X | ***H. FELIS* INFECTION, 6 MONTHS, 60X**

FIGURE 6.9 SDF-1 AND CXCR4 UPREGULATION IN THE STOMACH AFTER *HELICOBACTER* INFECTION.

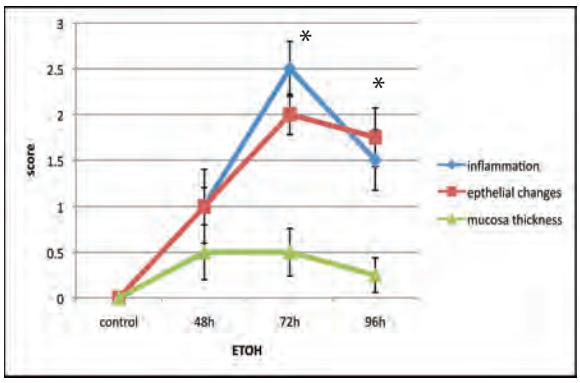
Gene expression is determined using real-time RT-PCR on total RNA from the stomachs of mice. Gene expression is represented in folds increase of gene expression over control group (1 fold), using β-actin as an internal control. **(A)** SDF-1 **(B)** CXCR4 Immunohistochemistry for CXCR4; **(C)** control mouse; **(D)** gastric mucosa of a mouse infected with *H. felis* for 6 months (arrow points a positive (brown) stained cell). Magnification, 60x. Counterstaining with hematoxylin.

FIGURE 6.10 ACUTE ALCOHOL INJURY CAUSES SEVERE INFLAMMATION AND SDF-1 UPREGULATION IN THE STOMACH

A



B



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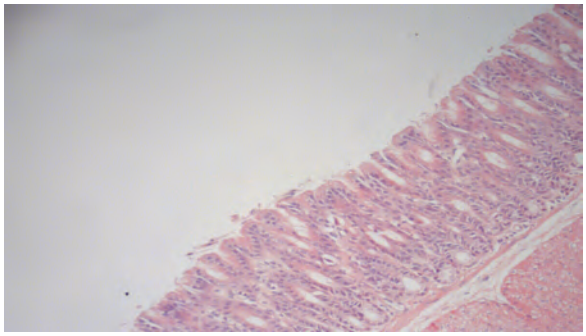
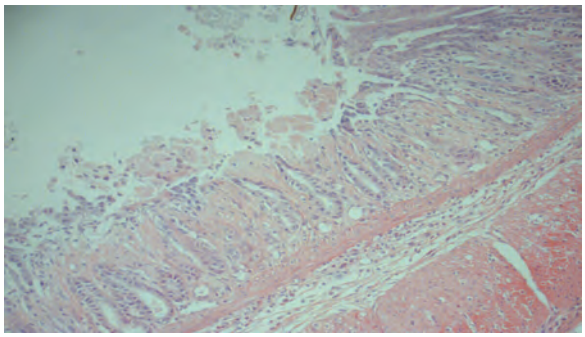


control mouse; 40x

D



alcohol, 96 hours; 40x

FIGURE 6.10 ACUTE ALCOHOL INJURY CAUSES SEVERE INFLAMMATION AND SDF-1 UPREGULATION IN THE STOMACH.

(A) SDF-1 expression was determined using real-time RT-PCR on total RNA from the stomachs of mice. Control mice did not receive an alcohol dose. Alcohol (5 gm/kg) was given once at 24h, 48 h, 72h and 96h respectively. Expression is represented in folds increase of gene expression over control group (1 fold). *$p<0.001$. **(B)** Mucosa injury scoring in the stomach at the same time points as in **(A)**, based on analysis of inflammation, epithelial lesion changes and mucosa thickness (edema). *$p<0.05$. **(C)** Normal histology of a control mouse. **(D)** Histology of mouse stomach exposed to alcohol for 96 hours. Magnification, 40X. N=5.

positive and CD45 negative, as known in literature. After 12 months of culture we were able to isolate a MSC colony that had a fibroblast - like phenotype, which was not different from the fresh cultured MSC. When our MSC were tested for lineage-specific markers, like cytokeratin, desmin, CD31, S100, etc., the results showed that they are not committed to any lineage differentiation. In order to be able to keep track of these cells in vivo, they were stably transfected with RFP and GFP constructs. When injected subcutaneously into mice, our RFP positive MSC were able to grow into subcutaneous tumors that had a natural appearance of fibrosarcoma. Moreover, our cells were able to transdifferentiate into fat, bone, cartilage, epithelial and endothelial cell lineages of the host mouse, after receiving a subcutaneous injection with cells, proving their functional plasticity was preserved. By injecting these cells subcutaneously we are mimicking a continuous influx of MSC into the host. An intravenous injection of these cells results in a low frequency engraftment of the skin, GI tract, mesentery and genitourinary tract (220).

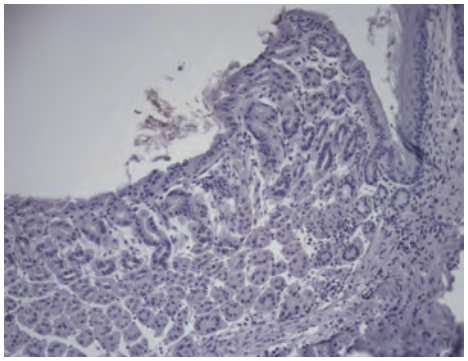
We have used these cells to study the homing of MSC into the stomach in a setting of chronic inflammation induced by *Helicobacter* infection. Our MSC are useful for this purpose because they resemble freshly cultured MSC, are not committed to differentiated lineages and preserved their plasticity in vivo.

RFP labeled MSC (MSC-RFP+) were injected subcutaneously into mice infected with *H. felis* for four months. The transdifferentiation of MSC into epithelial gastric cells occurred along with differentiation into submucosal non-epithelial cells, which was evaluated by direct fluorescence for RFP on frozen section (Fig. 6.11D and H), FISH for Y chromosome (green) costained with immunofluorescence for cytokeratin (red) (Fig.

FIGURE 6.11. RED FLUORESCENT POSITIVE (RFP) POSITIVE MESENCHYMAL STEM CELLS (MSC) HOME AND DIFFERENTIATE INTO GASTRIC EPITHELIAL CELLS.

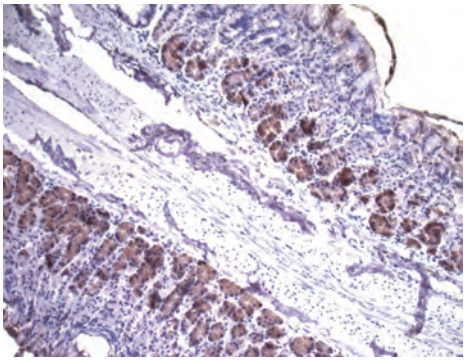
IMMUNOHISTOCHEMISTRY FOR RFP ON STOMACH

A



INFECTED MOUSE, CONTROL

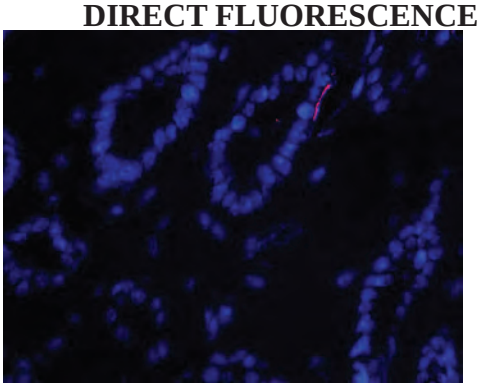
B



INFECTED MOUSE + MSC

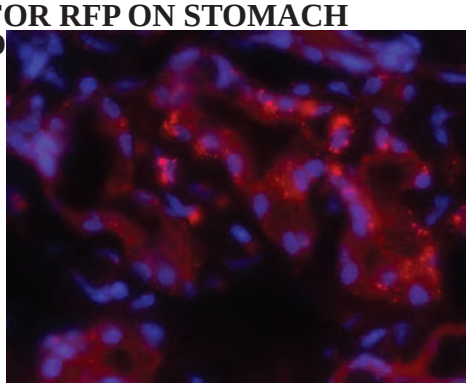
DIRECT FLUORESCENCE FOR RFP ON STOMACH

C



INFECTED MOUSE, CONTROL

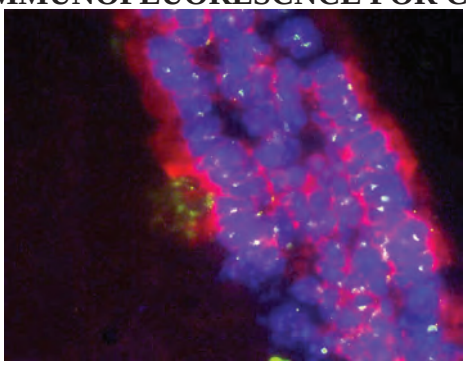
D



INFECTED MOUSE + MSC

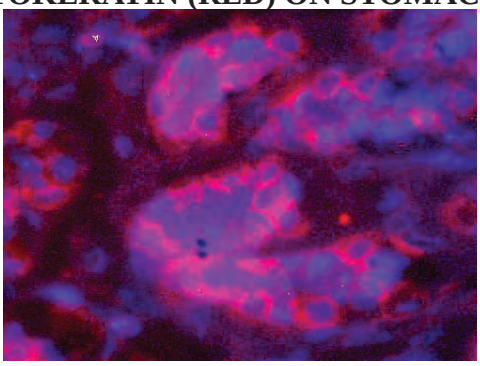
COLOCALIZATION OF FISH FOR Y-CHROMOSME (GREEN) AND IMMUNOFLUORESCNCE FOR CYTOKERATIN (RED) ON STOMACH

E



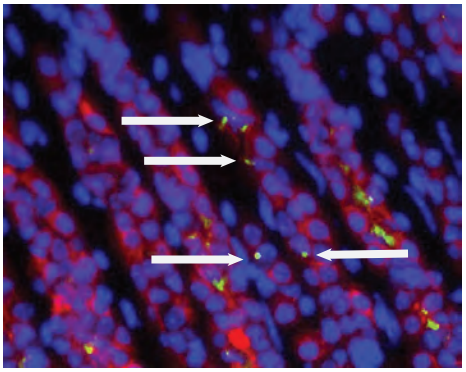
POS CONTROL FOR Y, MALE

F



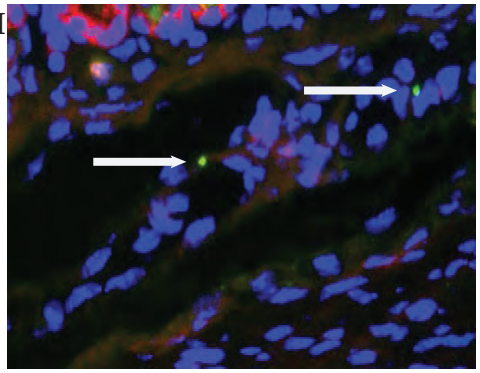
NEG CONTROL FOR Y, FEMALE

G



INFECTED MOUSE + MSC

H



INFECTED MOUSE + MSC

FIGURE 6.11 RED FLUORESCENT PROTEIN (RFP) POSITIVE MESENCHYMAL STEM CELLS (MSC) HOME IN THE STOMACH AND DIFFERENTIATE INTO GASTRIC EPITHELIAL CELLS.

Immunohistochemistry for RFP on stomach tissue. **(A)** Mouse infected with *H. felis* for 6 months, but not injected with MSC; **(B)** Infected with *H. felis* for 6 months that was injected with RFP positive MSC**.** Positive RFP immunohistochemistry staining is seen on stomach tissue (brown color). Counterstaining was done with hematoxylin.

Direct fluorescence on stomach tissue. **(C)** Negative for RFP of a mouse infected with *H. felis* for 6 months, but was not injected with MSC. **(D)** Positive for RFP on a mouse infected with *H. felis* for 6 months, which was injected with MSC. Counterstaining done with DAPI, cell nuclei are stained with blue.

FISH for Y chromosome. **(E)** Male mouse stomach. Y chromosome is stained in green, seen in most of the glandular cells. **(F)** Female mouse stomach is negative control for Y. Y chromosome staining is not seen in any of the in any of the glandular cells. Nuclei stained with DAPI in blue and cytokeratin stained in red. **(G)** Female mouse stomach infected with *H. felis* for 6 months and injected with MSC (male). Arrows point to Y positive cells in the glandular structures of the stomach. **(H)** Female mouse stomach infected with *H. felis* for 6 months and injected with MSC. Arrows point to Y positive cells in the submucosa of the stomach. These cells are not epithelial in origin, therefore negative for cytokeratin (red). Magnification, A and B (40X); C, D, E, F, G, H (60X).

6.11G and H) and immunohistochemistry for RFP on paraffin sections (Fig 6.11B). Control infected mice that did not receive injected cells were negative for RFP by immunohistochemistry (Fig 6.11A) and Y chromosome (Fig 6.11F). These results prove the ability of the MSC to home and transdifferentiate into stomach mucosal cells of both, epithelial and nonepithelial lineages.

10. MSC express a functional CXCR4, which is knocked down after their transfection with short hairpin CXCR4 constructs.

In order to assess the role of the SDF-1/CXCR4 axis in the pathogenesis of *Helicobacter* induced disease we knocked expression of CXCR4 down, by transfecting our MSC-RFP+ cells with short hairpin RNA for CXCR4 or its scramble control. Total of three different RNAi clones were used and, transfection efficiency was assessed using GFP fluorescence, which they encode.

FACS analysis of the MSC-RFP+ shows that they express CXCR4 (Fig. 6.12G, blue histogram), also conformed by western blotting (Fig. 6.12H, lane 1).

MSC-RFP+ cells were transfected with shScramble construct (control vector, named shScramble cells) or sequentially with shCXCR4 clone 1, 2, and 3 (called from here on shCXCR4 cells) constructs. Fluorescent microscopy of live cells confirmed that both cells lines were efficiently transfected with GFP and preserved their RFP positivity in vitro (Fig. 6.12B, C, E and F). Western blot analysis of the same cell lines, shows the strongest CXCR4 down regulation of the MSC transfected with all three clones of shCXCR4 (Fig. 6.12G, last lane).

FIGURE 6.12 MSC EXPRESS A FUNCTIONAL CXCR4, WHICH IS KNOCKED DOWN AFTER THEIR TRANSFECTION WITH SHORT HAIRPIN CXCR4 CONSTRUCTS.

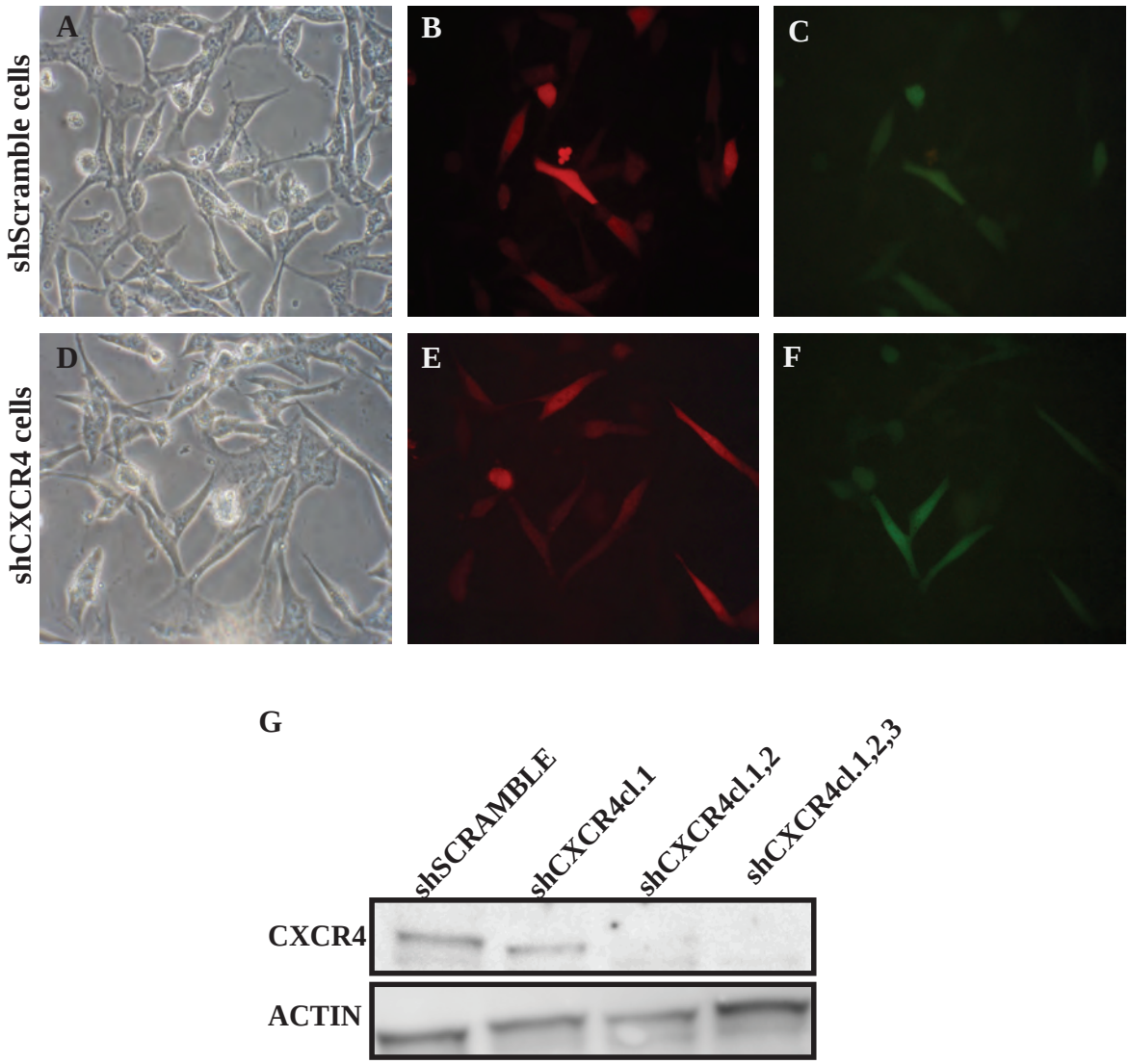


FIGURE 6.12 MSC EXPRESS A FUNCTIONAL CXCR4, WHICH IS KNOCKED DOWN AFTER THEIR TRANSFECTION WITH SHORT HAIRPIN CXCR4 CONSTRUCTS.

(A) shScramble MSC viewed under light microscopy, they are RFP positive **(B)** and acquired a GFP signal **(C)** after their cotransfection with the shScramble plasmid. **(D)** shCXCR4 MSC cells viewed under light microscopy, without any morphological changes observed, they preserved their RFP positivity **(E)** and gained a GFP signal **(F)** after a cotransfection with three different clones of shCXCR4. **(G)** WB for CXCR4 on total protein extracts from shScramble cells, MSC transfected with shCXCR4 clone1, shCXCR4 clone 1 and 2, and shCXCR4 clone1 and 2 and 3. β-actin was used as a loading control for each sample. Magnification, 20X.

11. SDF-1 stimulated MSC reduce their cAMP levels and promote their migration in a Transwell system.

SDF-1 stimulation of CXCR4 results in activation of G_i protein, which will inhibit the adenylate cyclase function, therefore resulting in a reduction of cyclic AMP (cAMP) levels in the cell. Stimulation of MSC-shScramble cells resulted in downregulation of cAMP by almost 50%, which was statistically significant ($p<0.00006$) (Fig 6.13A). On the other hand, the knock down CXCR4 cell line had a statistically insignificant change in cAMP levels ($p<0.23$), which confirms its inability to trigger CXCR4 signaling (Fig. 6.13A).

Measuring MSC migration through a Transwell system towards a SDF-1 gradient in vitro further tested CXCR4 functionality. An overnight stimulation of MSC-shScramble cells with 100ng/ml of SDF-1 resulted in a 200% increase in migration through the membrane when compared to migration of nonstimulated cells, which occurs by gravity (Fig. 6.13B and C). When shCXCR4 cells were stimulated in the same conditions, there were no changes in migration conforming a nonfunctional CXCR4 receptor (Fig. 6.13B and C). Therefore, CXCR4 knockdown MSC (shCXCR4 cells) could be used as a "loss of function" model for addressing gastric mucosal homing.

12. MSC homing in acute gastric inflammation is dependent on CXCR4 activity

In order to study homing of MSC to the gastric mucosa, we employed an acute injury model which allowed us to precisely time the injection of circulating cells with the

FIGURE 6.13 shCXCR4 EFFECTIVELY BLOCKS CXCR4-SDF-1 MEDIATED cAMP REDUCTION AND PREEVENTS CELL MIGRATION.

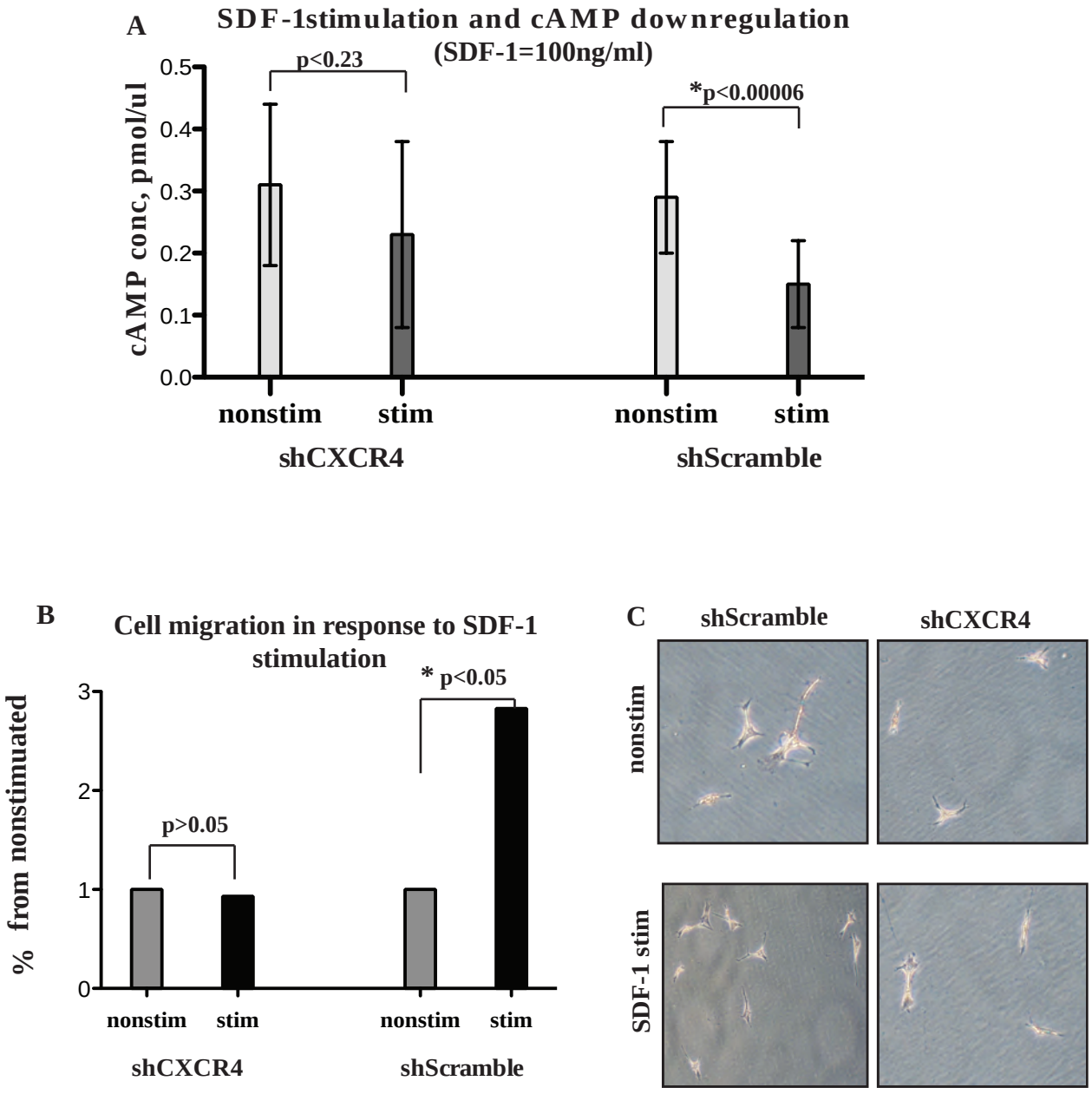


FIGURE 6.13 shCXCR4 EFFECTIVELY BLOCKS CXCR4-SDF-1 MEDIATED cAMP REDUCTION AND PREVENTS CELL MIGRATION

shCXCR4 or shScramble MSC were stimulated with 100ng/ml of SDF-1 for 10 min and cAMP was measured by ELISA.

(A) shScramble cells down regulated cAMP by almost 50%, which was statistically significant; (* $p<0.00006$). shCXCR4 cells decreased cAMP concentration by less then 30%, but failed to reach a statistical significance. ($p<0.23$). shCXCR4 or shScramble MSC were stimulated with 100ng/ml of SDF-1 for 12 hours and migrated cells were counted by Giemsa staining on the bottom of the plate.

(B) shScramble cells were able to migrate by almost 200% of its nonstimulated control. (* $p<0.05$). shCXCR4 failed to migrate under the same conditions. ($p>0.05$). **(C)** shScramble and shCXCR4 cells counted on the bottom of the plate. Magnification 20X. All experiments repeated 3 times.

availability of adequate SDF-1 levels. Mice were given ethanol at a dose of 5mg/kg of mouse body weight by oral gavage every 24h 4 times. The control group was given PBS of the same volume (200 μl). Because SDF-1 levels peaked at 72 hours after the first injection (Fig. 6.10B and D), we chose to give intravenously via a tail vein 1 million of either shScramble or shCXCR4 cells at 6 hours before the 3rd dose of ethanol. Animals were sacrificed 12h after the cells were injected. Cells with a functional CXCR4 (shScramble cells) were recovered in the gastric mucosa using PCR for RFP (alcohol group - 5.6 folds ($p<0.05$) and no alcohol group - 4.3 folds ($p<0.05$), while the cells with a nonfunctional CXCR4 (shCXCR4 cells) failed to produce a positive PCR. Control mice, with or without alcohol administration, did not have any RFP expression (Fig. 6.14A).

Gastric mucosa was also evaluated by direct fluorescence microscopy for RFP. The results supported the findings in the RFP PCR experiment. That is, mice that were injected with shScramble cells had RFP positive cells in the gastric mucosa, while shCXCR4 injected cells were not found by direct fluorescence in the mice stomach (Fig. 6.14B, C, D and E). We did not look into the phenotype of the homed MSC in the stomach, since more then 12 hours are needed for these cells to differentiate into epithelial lineages.

FIGURE 6.14 MSC REQUIRE A FUNCTIONAL CXCR4 RECEPTOR FOR HOMING IN THE GASTRIC MUCOSA.

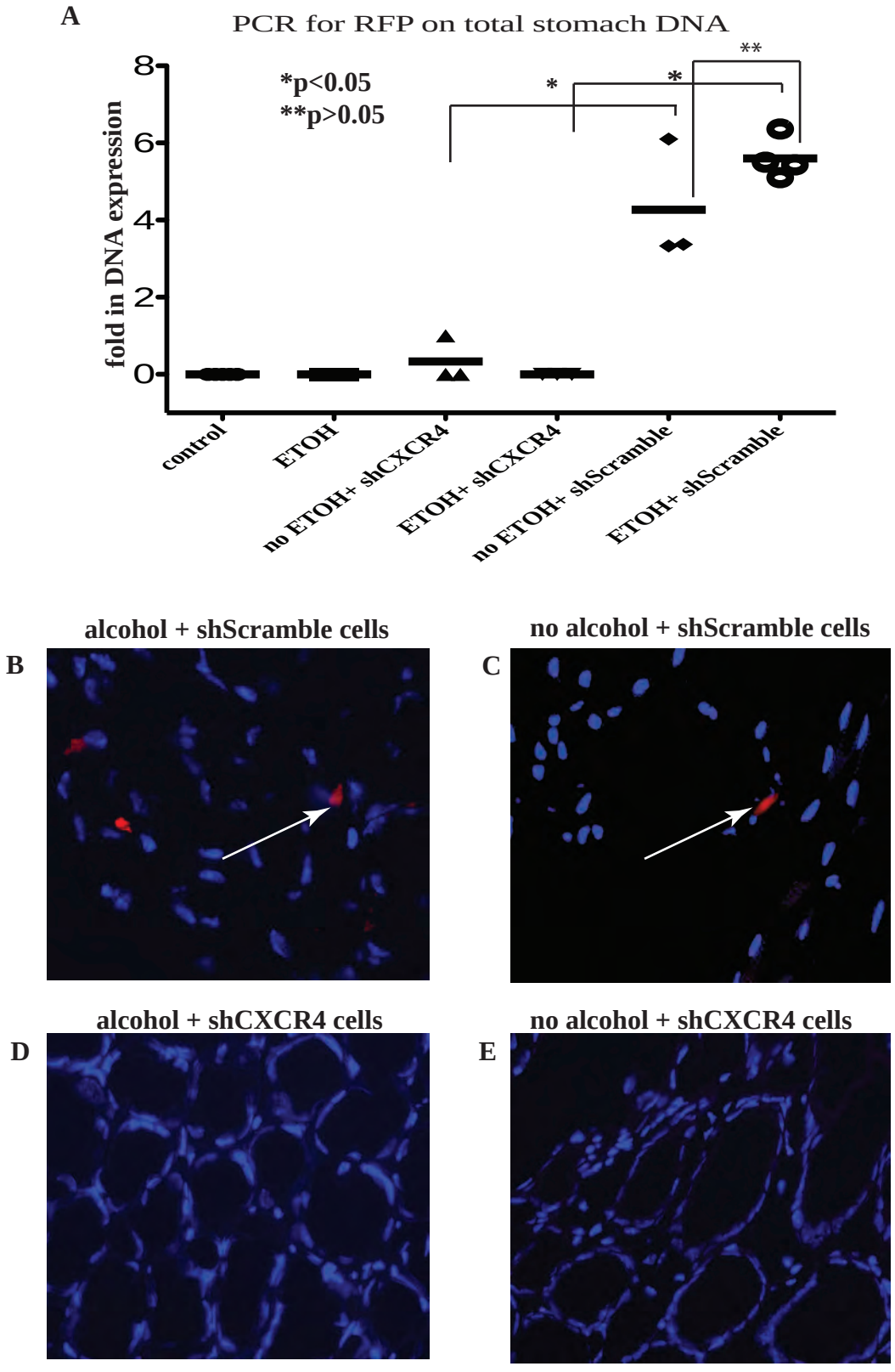


FIGURE 6.14 MSC REQUIRE A FUNCTIONAL CXCR4 RECEPTOR FOR HOMING IN THE GASTRIC MUCOSA.

Real time PCR for RFP on total stomach DNA: **(A)** Positive signal was detected in the shScramble groups, with or without alcohol. (*$p<0.05$) and (**$p>0.05$). N=3-5 animals per group. Statistical analysis was done using a nonparametric analysis (Mann-Whitney test). Direct fluorescence for RFP: **(B)** Positive cells (red) in the stomach mucosa of mice injected with shScramble cells and alcohol. (**C)** Positive cells (red) in the stomach mucosa of mice injected with shScramble cells, without alcohol. **(D)** and **(C)** no RFP cells detected.

Summary

The ability of BMDCs to differentiate into different embryonic lineages (ectoderm, mesoderm and endoderm) is supported by an overwhelming amount of publications (76, 77, 177-194). The inflammation of peripheral tissue, where the adult stem cells reside, causes an overexpression of inflammatory mediators (chemokines, cytokines) resulting in failure of the adult stem cell population. It is believed that the bone marrow's role is to repopulate the adult tissues with new progenitors, thus mediating a repair and a reconstruction process. It is unclear what would happen to the stem cell fate when exposed continuously to a chronic inflammatory environment.

The theory of cancer stem cells implies that many tumors rise from a stem cell, which is able to differentiate into all cell types of the tumor and self-renew, just like a tissue stem cell. Because cancer is considered to be a disease of unregulated self-renewal, the understanding of stem cell self-renewal may facilitate our understanding of cancer cell proliferation. Cancer stem cells along with the normal stem cells share self-renewal ability; one may extrapolate that cancer stem cells and normal stem cells use the same pathways and regulation mechanisms to control cell division. Indeed, the Notch and Wnt signaling pathways regulate normal stem cell self-renewal, and are also implicated in carcinogenesis.

Here, I use *H. pylori* infection to explore the interaction of the inflammatory environment within the gastric tissue with a stem cell, emphasizing on how the gastric

stem cell responds to inflammation. We believe that in response to chronic *Helicobacter* infection MSC can home to and engraft into the gastric mucosa, contributing over time to metaplasia, dysplasia, and cancer. We now recognize that a peripherally located stem cell (rather than a terminally differentiated cell) is the target of transformation; this may be the cancer stem cell or tumor-initiating cell that is responsible for cancer growth.

CXCR4/SDF-1 axis is an important tool used by MSC for homing. MSC not only express CXCR4, but also respond with migration and internal signaling when stimulated by SDF-1. Here, we showed how a functional CXCR4 is necessary for homing of MSC into the gastric mucosa, while the loss of function does not result in homing.

Next, we are going to test if homing is a CXCR4 dependent process in the setting of chronic *Helicobacter* infection using ore mouse model. We are going to infect mice with *H. felis* and inject them with shScramble or shCXCR4 cells, and then analyze their stomachs at different time points (2, 4, 6, 12 months) for engraftment and differentiation of MSC into epithelial cancer cells of the stomach. Based on our preliminary conclusions drawn from the homing experiment with alcohol-induced inflammation, we hypothesis that CXCR4-SDF-1 axis is indeed necessary for MSC to initiate the gastric adenocarcinoma in our mouse model of disease.

The concept that epithelial cancers can arise from BMDCs greatly alters our overall understanding of cancer initiation and progression and has broad implications for the development of anticancer therapies. The focus of anticancer cure, based on our findings, has to focus also on treating chronic inflammation, eliminate the causes of chronic inflammation and preventing a normal stem cell become a cancer stem cell.

CHAPTER VII. DISCUSSION

The host immune response plays a critical role in determining disease manifestations of chronic infections. Inadequate immune response may fail to control infection, although in other cases the specific immune response may be the cause of tissue damage and disease. More than one organism infects the majority of patients with chronic infections, yet the interaction between multiple active infections is not known, nor is the impact on disease outcome clear. Using the BALB/c strain of mice, we showed that *Toxoplasma gondii* infection in a host infected with *Helicobacter felis* alters the natural outcome of *T. gondii* infection, allowing uncontrolled tachyzoite replication and severe organ damage. In addition, infection with *T. gondii* alters the specific *H. felis* immune response, converting a previously resistant host to a susceptible phenotype. Dual-infected mice had developed a skewed immune response towards a Th1 phenotype. These changes were associated with severe gastric mucosal inflammation, parietal cell loss, atrophy, and metaplastic cell changes. These data demonstrate the profound interactions between the immune response to unrelated organisms, and suggest these types of interactions may impact clinical disease.

The interaction between different microbes in the same host has an important implication for the human pathology. The prime example is the HIV (human immunodeficiency virus) infection and other viruses, like Hepatitis C or B, Epstein Bar virus (EBV), cytomegalovirus virus (CMV), and human T-cell lymphotropic virus. It is well known that coinfection with hepatitis C virus (HCV) and HIV is associated with increased HCV replication and a more rapid progression to severe liver disease, including the development of cirrhosis and hepatocellular carcinoma (221). Coinfection of HIV and EBV leads to a higher risk of developing lymphoma in humans. The coinfection of

helminthes and viruses has a great deal of importance as well. Furze et al. have described how the coinfection with *Trichinella spiralis* ameliorates the clinical picture of influenza virus (222); on the other hand ascaridosis protects against cerebral and blood-stage malaria (223, 224). It is very important to be aware of all coinfections that affect the human body as this may lead to improved clinical outcomes when treating against one pathogen.

To further elucidate the role of Th1 cytokines and their regulation in *Helicobacter* induced inflammation, we used C57BL/6 mice to identify T-bet as a central regulator of the cytokine environment during infection. Despite sustained infection, T-bet KO mice responded with a blunted Th1 response associated with preservation of parietal and chief cells and protection from the development of gastric cancer. Unexpectedly, T-bet KO mice develop a gastric environment that would not be expected based on the phenotype of T-bet KO CD4 cells alone, but also to NK cells, that produce a non T-bet dependent IFNγ. Activity of T-bet modulates the expression of the key gastric mucosal cytokines (IFNγ, TNF-α and IL-1β) associated with gastric cancer and may be a target for therapy to restore immune balance clinically in patients at risk for gastric cancer. Studying the presence of single nucleotide polymorphisms (SNP) (225) in the human T-bet gene may provide additional clues in determining how host genetics influences the disease outcome.

There is evidence that Fas signaling may have a role in proliferation, and in some cases function as an oncogene (80). Because gastric mucosal cells acquire many characteristics of immune cells during *Helicobacter* infection, including cytokine

production (226) and the capacity to present antigens (227), we addressed whether cells within the infected gastric epithelium possess MHCII/Fas Ag interactions similar to those found in lymphocytes and/or dendritic cells. We showed that MHCII molecules inhibit Fas-mediated signaling in gastric mucosal cells through inhibition of receptor aggregation and prevention of downstream caspase activation by an actin cytoskeleton-dependent mechanism. Under conditions of self-limited inflammation, MHCII expression by gastric mucosal cells may be beneficial. Indeed, presentation of antigens by gastric mucosal cells and binding of *Helicobacter* organisms directly to the MHCII complex (120, 123) have been reported and potentially play a role in coordinating the local immune response to infection. However, the immune response is ineffective, and failure to eliminate the organism results in a continued inflammatory state. Under these conditions, MHCII expression may become detrimental. The inhibition of apoptosis due to MHCII involvement may be one mechanism by which abnormal clones of cells avoid apoptosis and may help explain the progression of the metaplastic cell lineage to adenocarcinoma. Retrospective studies suggest that differences in patient susceptibility to gastric cancer may be linked to the MHC locus [for example, expression of the MHC DQA1*0102 allele is associated with a decreased risk of intestinal-type gastric adenocarcinoma (228)], offering the exciting possibility of further identifying the genetic compositions of patients most at risk for gastric cancer and offering targeted intervention based on these signaling alterations.

While it is widely accepted that the cancer stem cell derives from a peripheral tissue stem cell pool, there is accumulating evidence from mouse and human studies that mobilized pluripotent stem cells originating in the bone marrow may seed distant sites of

injury and act as the cancer stem cell (127, 220, 229-233), and may contribute to tumor stroma as fibroblasts, myofibroblasts and endothelial cells (234-238), and provide crucial signals to the tumor stem cells. The pressing questions therefore remain. What is it about the inflammatory environment that encourages the malignant growth of peripheral organ stem cells? What are the environmental factors, which call in circulating pluripotent stem cells, allowing them to become established within injured tissue?

Within the bone marrow are at least two populations of stem cells, hematopoietic and mesenchymal stem cells. The hematopoietic stem cell is responsible for producing all the formed elements of the blood. The mesenchymal stem cell was originally recognized as essential for the production of stromal support cells necessary for hematopoiesis, and later recognized as having tri-lineage potential, with the ability to differentiate to bone, cartilage and fat. More recently the mesenchymal stem cell has been shown to possess the plasticity to differentiate down most all cell lineages in the body and participate in tissue restoration and healing as epithelial cells as well as stromal cells. Under normal physiologic conditions, multiple types of epithelial cells have been shown to be derived from bone marrow cells including epithelium of the lung, gastrointestinal tract and skin (76). Single bone marrow derived stem cells have been shown experimentally to expand within the host and transdifferentiate into diverse epithelial lineages. These data strongly support the existence of a single pluripotent stem cell rather than multiple committed progenitor cells as the cell of origin (173).

Within the gastrointestinal tract, isolated BM-derived epithelial cells in the gastric pits of the stomach, the small intestinal villi, the colonic crypt, and rarely in the esophagus appear as single differentiated epithelial cells, and do not appear to engraft

into the stem cell niche. These cells can be recovered months after transplantation so it appears that either the cells are long lived, or engraftment is an ongoing process. Their role within the peripheral tissue is not clear. Human studies, which take advantage of patients receiving gender-mismatched organs/marrow, also demonstrate this phenomenon on BMDCs residing in the peripheral tissues as tissue specific cells. In these studies, BMDCs have been found in increased numbers within the inflamed epithelium, and the level of engraftment correlates to some degree with the level of graft verses host disease present (239, 240).

To evaluate BMDCs activity in an inflammatory model, we used the well-described *H. felis*/C57BL/6 mouse model of gastric inflammation and injury (97, 102). The C57BL/6 model of *Helicobacter felis* induced gastric cancer is ideal to address this question because C57BL/6 mice do not develop gastric cancer under control conditions, but they reliably develop cancer with *Helicobacter felis* infection (127, 174). The process and pathological changes within the stomach recapitulate human disease, where gastric cancer in the absence of *Helicobacter* infection is unusual, while longstanding infection carries a significant (up to 1-3%) risk of gastric cancer (17) making this a very useful animal model. For these studies, C57BL/6 mice transplanted with marked bone marrow were infected with *Helicobacter felis*, and evaluated at various time points for the presence of BMDCs within the gastric mucosa. Engraftment of BMDCs and of culture adapted MSC within the mucosa, and differentiation to an epithelial cell phenotype first becomes evident at about 20 weeks of infection, corresponding with the appearance of metaplastic cell lineages. As time progresses, the number of BMDCs-glands increases dramatically suggesting both an expansion of resident BMDCs through proliferation,

and/or the recruitment of additional cells (127). Based on these studies, we believe long-standing inflammation and inflammatory mediated damage is required for BMDCs engraftment within the gastric epithelium. Long standing inflammation and tissue remodeling is associated with premalignant and malignant conditions, further suggesting to us that this environment may be driving the transformation of the BMDCs within inflamed sites.

In addition to epithelial cells within the tumor, BMDCs also comprise a subset of cells within the tumor stroma and within seemingly uninvolved epithelium and subepithelial spaces adjacent to the tumors. Adipocytes, fibroblast, endothelial cells and myofibroblasts derived from bone marrow precursors can be found in areas adjacent to dysplasia and neoplasia.

For as many questions these studies answer, there are more that are raised, and remain to be answered. The data from the *H. felis* induced gastric cancer model implies that BMDCs are recruited to the inflamed tissue and there, transform. We do not yet know if BMDCs in the tissue regain access to the circulation setting up a scenario where environmental influences at one site could affect the BMDCs, which then could grow at a different site. If this were the case, it could be imagined that BMDCs that carry mutations could home to and engraft into peripheral tissues possibly resulting in malignancy in a shorter time frame, or with a more aggressive phenotype (Fig 7.1). Additional work in the animal model is needed to address these points.

The next dilemma that our hypothesis is facing is, what are mechanisms by which the BMDCs are able to mobilize and attach at the “inflammatory niche”? It is known from literature that CXCR4/SDF-1 axis is one of the most important tools used by

FIGURE 7.1 THE PROPOSED MODEL OF GASTRIC CANCER DERIVED FROM CXCR4 POSITIVE MESENCHYMAL STEM CELLS.

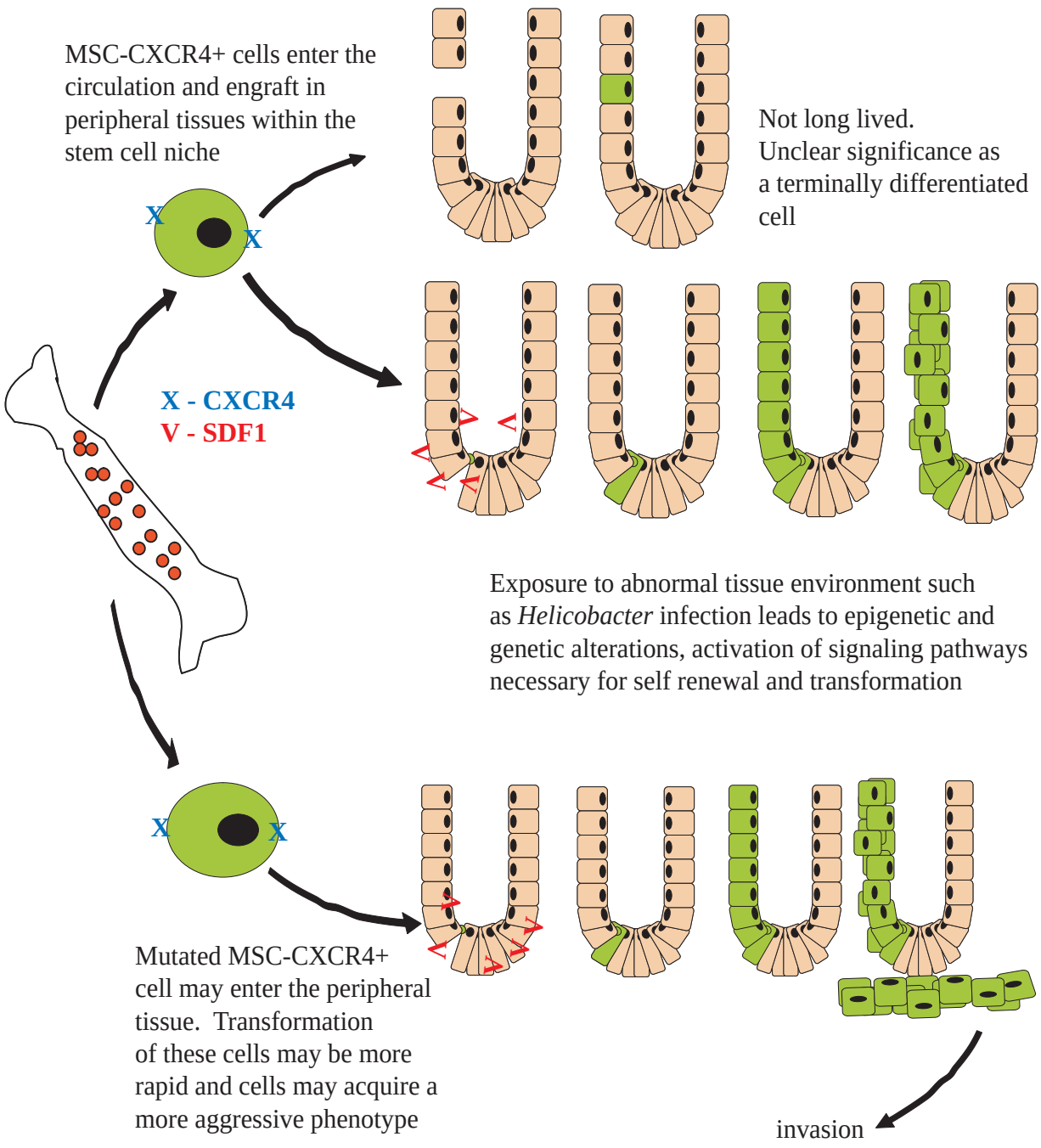


FIGURE 7.1 THE PROPOSED MODEL OF GASSTRIC CANCER DERIVED FROM CXCR4 POSITIVE MESENCHYMAL STEM CELLS.

Mesenchymal stem cells from bone marrow, which are positive for CXCR4, enter the circulation and engraft in the inflamed peripheral tissue that secret SDF1 within the stem cell niche. If inflammation is not chronic and short lived, these cells will aid with the local repair by transdifferentiation into a terminally differentiated stomach mucosa cell. If the inflammation perpetuates and becomes chronic, either an already mutated MSC or a locally mutated stem cell acquires a mutagenic phenotype, giving rise to neoplasia. The later process is also dependent on the CXCR4/SDF-1 axis.

mesenchymal stem cells to navigate through to a targeted destination, like an inflammatory site. MSC express CXCR4 physiologically, allowing them to migrate in vivo and in vitro. Using an acute alcohol injury model in mice we were able to conform that a functional CXCR4 receptor is necessary for homing of MSC into the gastric mucosa. The “CXCR4 loss of function” model was not tested in our *Helicobacter* model of inflammation, but we expect that homing, followed by engraftment of MSC in the stomach would be at least diminished. While the CXCR4/SDF-1 axis seems to be an important part of the homing process, it may not be the only tool that is used by the stem cells.

Recently, more molecules that facilitate the mobilization of MSC were described. A role for galanin in mesenchymal stem cells migration, probably through activation of the GalR2 (galanin receptor-2) receptor, was described by Louridas et. al (241). MSC express both, galanin and its receptors; they are able to mobilize in response to galanin in vitro using a “wound assay” and in vivo, by homing in the bone marrow of a galanin transgenic mouse.

Zisa et al. have described how intramuscular VEGF is inducing recruitment of stem cells into the heart and skeletal muscle hence reducing the cardiomyopathy in a hamster model of heart failure (242). Their results show that VEGF was able to recruit also CXCR4 positive progenitors along with c-kit positive cells. There may be a correlation between the VEGF and CXCR4-SDF-1 axis in homing of MSC?

Hoxa3 is a transcription factor that is known to promote angiogenesis during tissue repair and accelerates cutaneous healing in vivo. Mace et al. group have

demonstrated how a skin wound that overexpresses Hoxa3 attracts more bone marrow derived cells and improves the rate of healing in mice (243).

Matrix metalloproteinase one (MMP1) and its cognate receptor PAR1 were studied by Ho et al. and determined that MMP1/PAR1 axis is an important mean for MSC mobilization towards a glioma tumor microenvironment in vivo and in vitro (244). Their model resembles our model of gastric cancer environment attracting in stem cells for repair.

The field of cancer biology has undergone significant shifts in recent years, with a focus on identifying and understanding the cancer stem cell. The evolving picture is one of a rare group of cells within a tumor with a surface marker profile and gene expression pattern unique to its role as a stem cell. A body of work suggests that the cancer stem cell may arise from the bone marrow; however, more work is needed to determine if this notion can be extrapolated to other models of cancer. In addition the importance of BMDCs in human cancer needs to be clarified. Characterizing and developing markers for the BMDCs subpopulations that can contribute to solid tumors is of major importance to facilitate early detection in patients and to develop targeted therapies aimed at the cancer stem cell. It seems likely that some solid tumors may be derived from resident tissue stem cells rather than circulating BMDCs, with the environment, genetic make up of the host and other factors dictating the cell that undergoes transformation. It is extremely important for these two paradigms to be understood- in their similarities, as well as their differences. The possible role of cell fusion by BMDCs has yet to be fully elucidated and research in this area will likely provide novel and exciting insights into the complexity of the relationship of the BMDCs and peripheral tissues. Additionally, the

concept of an aberrant stem cell niche and its role in contributing to cancer development from BMDCs will need further clarification. Prevention and/or reversibility of preneoplastic lesions (for example by eradication of *H. pylori*) are clearly worth addressing further in the *Helicobacter* mouse model and can be considered in the context of the biology of stem cell differentiation.

Finally, the precise relationship of chronic inflammation to BMDCs needs further investigation. One suspects that future studies will reveal the involvement of many cell types and specific factors – including cytokines, chemokines, hypoxia and oxidative stress – in the mobilization, recruitment, engraftment, and progression of stem cells toward cancer. Understanding these mechanisms offers the exciting hope of new approaches to cancer treatment.

CHAPTER VIII. REFERENCES

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